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Greater free plasma VEGF and lower soluble VEGF receptor-1 in Acute Mountain Sickness.

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Abstract

Vascular endothelial growth factor (VEGF) is a hypoxia-induced protein that produces vascular permeability, and limited evidence suggests a possible role for VEGF in the pathophysiology of acute mountain sickness (AMS), and/or high altitude cerebral edema (HACE). Previous studies demonstrated that plasma VEGF alone does not correlate with AMS; however, soluble VEGF receptor (sFlt-1), not accounted for in previous studies, can bind VEGF in the circulation, reducing VEGF activity. In the current study, we **hypothesized** that free VEGF is greater and sFlt-1 less in subjects with AMS as compared to well individuals at high altitude. **Methods:** Subjects were exposed to 4300 m for 19-20 hours (baseline 1600 m). The incidence of AMS was determined using a modified Lake Louise symptom score and the Environmental Symptoms Questionnaire for cerebral effects. Plasma was collected at low altitude and after 24 hours at high altitude, or at time of illness, and then analyzed by ELISA for VEGF and for soluble VEGF receptor, sFlt-1. **Results:** AMS subjects had lower sFlt-1 at both low and high altitude as compared to well subjects, and a significant rise in free plasma VEGF on ascent to altitude compared to well subjects. **Conclusion:** We conclude that increased free plasma VEGF on ascent to altitude is associated with AMS, and may play a role in pathophysiology of AMS.

Introduction

Acute exposure to high altitude can result in acute mountain sickness (AMS), the hallmark of which is headache (7). AMS can progress to high altitude cerebral edema (HACE), a more severe and often deadly form of altitude illness thought to be due to leakiness of the blood brain barrier (7). High altitude hypoxia stimulates expression of vascular endothelial growth factor (VEGF)(5), which increases vascular permeability and produces brain edema in hypoxic animal models (3, 4, 10, 19). In addition, blocking VEGF prevents hypoxic brain edema (19). VEGF therefore deserves consideration as a mechanism to explain the vascular leak of AMS/HACE.

Previous studies investigating the role of VEGF in AMS found no significant correlation of plasma or serum VEGF with AMS (15, 22). However, recent work shows that hypoxia also stimulates expression of a circulating, soluble VEGF receptor, soluble fms-like tyrosine kinase receptor-1 (11). The soluble receptor, known as soluble Flt-1 (sFlt-1), binds circulating VEGF thereby reducing VEGF-induced vascular leak and angiogenesis (11). Because the presence of sFlt-1 can have significant impact on VEGF-induced vascular leak, plasma sFlt-1 as well as VEGF concentration needs to be measured to assess the role of VEGF in AMS. We hypothesized that plasma sFlt-1 concentration would be less and free VEGF concentration therefore greater in subjects developing AMS compared to well subjects following acute exposure to high altitude.

Our approach was to expose subjects to acute hypobaric hypoxia by rapid ascent to 4300 m (Pikes Peak, Colorado) from 1600 m. We also measured plasma EPO concentrations since EPO is a biochemical marker of altitude stress (6).

Materials and Methods

Study Design. Approval from the Mesa State College Human Research Committee and the Colorado Multiple Institutional Review Board (COMIRB) at the University of Colorado Health Sciences Center was obtained to perform this study. Informed consent was obtained from 20 subjects in accordance with NIH guidelines. Subjects dropping out of the study were not replaced and subjects could drop out at any time without penalty. All subjects were healthy adults who resided at an elevation of 1370 m to 1645 m. Exclusion criteria included pregnancy, altitude exposure duration more than 24 h above 2100 m within 2 weeks of the study. None of the subjects had a prior history of AMS. Subjects were originally enrolled into a study that was designed to compare placebo to two drugs for AMS prevention. However, a loss of data and blood samples in the drug treatment groups led to them not being included in this study. The elevation of residence will be referred to as low altitude throughout this manuscript. Subjects were driven to 4300 m (Pike's Peak summit) over 2 hours by car and stayed overnight. Plasma was collected from subjects at low (1370 m or 1645 m) and high (4300 m) altitudes. High altitude blood draws occurred after 19-20 hours at high altitude or, if the subject was severely ill with AMS and treatment was required (IV dexamethasone and oxygen), a venous blood draw was performed just prior to treatment. Plasma was used for analyses of VEGF, sFlt-1 and EPO. Of the 20 subjects recruited, data from one subject could not be evaluated due to hemolysis during sample preparation at high altitude.

Acute Mountain Sickness. The Symptoms Questionnaire (ESQ-III short form) was completed before ascent and either after 19-20 hours at altitude or when removed from the study as a result of severe AMS. An ESQ-III $\geq$ 0.7 *and* a Lake Louise Score of $\geq$ 3, with a headache present, were required for diagnosis of AMS (13).

Plasma EPO, VEGF, sFlt-1. Whole blood was collected into Vacutainer™ tubes containing EDTA as an anticoagulant and centrifuged at 26^0 C for 30 min at 400x*g*. Plasma was withdrawn, aliquoted to 0.5 ml, flash frozen in liquid nitrogen and stored at –80°C until use. ELISA kits were used for evaluation of plasma EPO, VEGF and sFlt-1 (R&D systems, MN, catalogue #s: DEP-00, DVE-00, DVR-100). When the coefficient of variance was less than 10% (n = 3 replicates per sample), data were considered reliable.

Free VEGF. The VEGF kit from R&D Systems has been shown to measure only VEGF not bound to sFlt-1 (1, 9, 16). Literature from R&D Systems in regard to VEGF ELISA kit DVE-00 reports no interference with sFlt-1 until greater than 1250 pg/ml. Previous reports using this kit have referred to plasma VEGF measured using this method as 'free VEGF'(12, 16); however, the method has never been tested to determine whether it identifies VEGF bound to another important plasma receptor, alpha-2-macroglobulin (2). We have tested this VEGF ELISA kit for cross reactivity with VEGF bound alpha-2-macroglobulin using methods identical to those used by R&D Systems to test for cross-reactivity with sFlt-1 and KDR. Varying concentrations of alpha-2-macroglobulin (10-250 ng/ml) were incubated with a mid-range VEGF standard for 1 hour at 37ºC, and then analyzed using the R&D Systems VEGF ELISA kit (DVE-00). There was a consistent

50% reduction in detectable VEGF in the presence of alpha-2-macroglobulin at all concentrations tested. Circulating VEGF has been shown to bind non-covalently with native alpha-2-macroglobulin, its binding affinity such that it remains in equilibrium with circulating VEGF (2), as demonstrated by our data.

VEGF measured decreased as alpha-2-macroglobulin increased in concentration, suggesting that the ELISA is not measuring VEGF bound to alpha-2-macroglobulin. Therefore, circulating VEGF measured by this ELISA kit is referred to as free plasma VEGF through out this manuscript. The ELISA for sFlt-1 measures total plasma sFlt-1, that which is bound to VEGF and unbound.

Statistics. Data comparing low to high altitude values were analyzed using paired student T-tests. Independent student T-tests were used to determine differences between well and AMS subjects at low and at high altitude. Correlations between VEGF, sFlt-1, EPO and SaO_2 were also determined. Significance was set at $p \leq 0.05$ for all statistical analyses.

Results

Of the 20 subjects in the study, sufficient plasma for all analyses was obtained at both altitudes in 19 subjects. The concentrations of plasma VEGF, sFlt-1 and EPO in well and AMS subjects are presented in Table 1. VEGF increased at high altitude in subjects who developed AMS but not in those who remained well. Although plasma sFlt-1 increased with ascent to high altitude in all subjects, concentrations were lower in the AMS as compared to well subjects at both low and high altitude**.** Erythropoietin (EPO) increased at high altitude in all subjects. Oxygen saturation was equivalent between well and AMS

subjects at high altitude, 86.33 ± 1.33 vs. 88.78 ± 0.983, respectively. At 4300 m, there was no correlation between oxygen saturation and plasma VEGF or sFlt-1, however EPO positively correlated with SaO_2, R2 = 0.31, p = 18.

Discussion

The main findings of this study are that subjects who developed AMS at high altitude had higher sFlt-1 levels at low and high altitude, and increased free plasma VEGF compared to those who remained well. Since subjects who subsequently developed AMS had low plasma sFlt-1 at low altitude as compared to those who did well, low altitude sFlt-1 concentration may be a predictor of AMS.

VEGF is a hypoxia-induced protein that can acutely increase vascular permeability and may contribute to the development of AMS/HACE by increasing cerebral capillary permeability (17). In our study, AMS subjects had a greater increase on ascent to altitude in VEGF available to bind endothelium and cause vascular leak as compared to well subjects. Previous reports indicating either no change in VEGF with ascent to altitude or a decrease in plasma VEGF that was not associated with AMS (15, 18), differed from the current study. Our study employed a more acute ascent profile than previous studies and each subject was used as his/her own control, allowing paired data analysis. Also, sFlt-1 bound to VEGF was not measured in previous studies in subjects exposed to high altitude.

Previous studies have clearly shown that sFlt-1 plays a role in various pathologies and can inhibit activity of circulating VEGF (8, 12, 14, 21). Soluble VEGF receptor, sFlt-1, is

an important factor when determining the pathophysiologic role of VEGF because it binds plasma VEGF and effectively reduces the amount VEGF available to bind endothelium (4). Oral administration of sFlt-1 reduces VEGF-induced vascular permeability (17). The current study indicates that sFlt-1 is greater and free VEGF is less in those who remain well as compared to those who develop AMS at high altitude. Further, plasma sFlt-1 was greater in well subjects at low altitude, *prior* to ascent to altitude as compared to those who developed AMS, suggesting sFlt-1 plasma concentration at low altitude may be a predictor of AMS. Interestingly, elevated sFlt-1 concentration has recently been reported as a putative predictor of preeclampsia, prior to clinical onset (12). Prior to clinical onset, preeclamptic patients had greater plasma sFlt-1 and less free VEGF than healthy pregnancies at similar gestational time points. VEGF, and the similar placental-like growth factor (PlGF), are crucial for normal pregnancy. Inducing transcription of sFlt-1 in rats produced preeclamptic-like disease, and subsequent exogenous administration of VEGF and PlGF rescued the animals from disease. These data indicate that circulating VEGF has significant impact on endothelial function that can be inhibited by binding to sFlt-1. Further studies are needed to clarify the correlation between plasma sFlt-1 and free VEGF at low altitude and when at high altitude.

One limitation of this study is that variation between samples was relatively large and potential differences between VEGF at low and high altitude in well subjects may not have been evident in this small sample size. Because whole blood was processed to plasma by the same personnel at both altitudes and equipment used to process blood at

low altitude was transported for use at high altitude, it is unlikely that experimental error was imposed by processing techniques at low vs. high altitude. Vacutainers were used to withdraw venous blood samples at both altitudes, however the lower barometric pressure at 4300 m reduced the efficiency of the vacutainers. For example, a 10 ml vacutainer only collected 4-6 ml blood at high altitude. Increased handling of the blood led to a large degree of hemolysis. Interestingly, the hemolysis occurred primarily in the drug treatment groups, with only one placebo sample hemolyzing at altitude. Subjects in this study were treated with a placebo because they were initially part of a larger study designed to investigate prevention of AMS by drug treatment. It is unlikely that the placebo had an effect on circulating VEGF, sFlt-1 or EPO concentrations.

Circulating VEGF has been shown to bind non-covalently with native alpha-2-macroglobulin and its binding affinity is such that it remains in equilibrium with circulating VEGF and probably does not alter VEGF function (2). There is no evidence to suggest that alpha-2-macroglobulin is altered by hypoxia. Because alpha-2-macroglobulin reduces VEGF detected by the ELISA kit used in this study (see Methods), it is unlikely that alpha-2-macroglobulin is responsible for the increased VEGF at altitude.

Interestingly, in this study oxygen saturation was not lower in subjects with AMS compared with those who remained well at high altitude. Further, although VEGF and sFlt-1 are hypoxia-sensitive proteins, circulating concentrations of neither protein were correlated with oxygen saturation. However, plasma concentrations of erythropoietin, also a hypoxia-sensitive protein, were negatively correlated with oxygen saturations at

high altitude, as has been previously reported (6). The effect of hypoxia on VEGF and sFlt-1 may be more sensitive to changes in oxygen content and delivery that to changes in oxygen saturation. Further research is needed to determine the mechanisms by which hypoxia influences circulating concentrations of VEGF and sFlt-1 in humans ascending to high altitude.

Overall, free plasma VEGF increased with ascent to altitude in subjects who developed AMS, while those who remained well at altitude had greater plasma sFlt-1 prior to and following ascent to high altitude. VEGF-endothelial interactions resulting in vascular leak can be inhibited by administration of exogenous sFlt-1 and vascular leak has been strongly implicated in AMS/HACE (8, 19, 23). We conclude that free VEGF may play a role in the pathophysiology of AMS.

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Table 1. Concentrations of circulating plasma factors in well subjects compared to those with AMS.

	Well (n = 10)		**AMS (n = 9)**	
	Low Altitude	High Altitude	Low Altitude	High Altitude
VEGF (pg/ml)	13.7 ± 13.5 (0 – 81.5)	135.6 ± 69.9 (0 – 432.6)	20 ± 17.5 (0 – 141.8)	196.2 ± 77# (0 – 466.5)
sFlt-1 (pg/ml)	22.3 ± 4.9* (0 – 43.3)	37.6 ± 4.6#* (25.5 – 50.68)	2.5 ± 1.6 (0 – 12.8)	17.1 ± 7.2# (0 – 59.4)
EPO (U/ml)	6.8 ± 1.9 (0.6 – 13.4)	29.8 ± 8.1# (0 – 78.5)	6.85 ± 1.15 (2.3 – 11.5)	30.13 ± 3.75# (15.9 – 51.2)
ESQ-C	0.02 ± 0.02	0.26 ± 0.06	0.11 ± 0.06	1.42 ± 0.19
LLSQ	0.27 ± 0.19	3.5 ± 0.3	0.5 ± 0.22	6.5 ± 0.31

ESQ-C, Environmental Symptoms Questionnaire – Cerebral, LLSQ - Lake Louise Symptoms Questionnaire # $p < 0.05$ High vs. Low, * $p < 0.05$ Well vs. AMS, data presented as mean ± SEM