Rat brain VEGF expression in alveolar hypoxia: possible role in high-altitude cerebral edema

FENGPING XU AND JOHN W. SEVERINGHAUS
Department of Anesthesia, University of California Medical School, San Francisco, California 94143-0542

**Xu, Fengping, and John W. Severinghaus.** Rat brain VEGF expression in alveolar hypoxia: possible role in high-altitude cerebral edema. J. Appl. Physiol. 85(1): 53–57, 1998.—The mechanism by which hypoxia causes high-altitude cerebral edema (HACE) is unknown. Tissue hypoxia triggers angiogenesis, initially by expressing vascular endothelial growth factor (VEGF), which has been shown to increase extracerebral capillary permeability. This study investigated brain VEGF expression in 32 rats exposed to progressively severe normobaric hypoxia (9–6% O2) for 0 (control), 3, 6, or 12 h or 1, 2, 3, or 6 days. O2 concentration was adjusted intermittently to the limit of tolerance by activity and intake, but no attempt was made to detect HACE. Northern blot analysis demonstrated that two molecular bands of transcribed VEGF mRNA (~3.9 and 4.7 kb) were upregulated in cortex and cerebellum after as little as 3 h of hypoxia, with a threefold increase peaking at 12–24 h. Western blot revealed that VEGF protein was increased after 12 h of hypoxia, reaching a maximum in ~2 days. The expression of flt-1 mRNA was enhanced after 3 days of hypoxia. We conclude that VEGF production in hypoxia is consistent with the hypothesis that angiogenesis may be involved in HACE.

angiogenesis; cytokines; brain capillary leak; acute mountain sickness

**HIGH-ALTITUDE CEREBRAL EDEMA (HACE)** is one form of severe acute mountain sickness. The pathophysiological link between hypoxia and HACE is poorly understood. Pathological findings include retinal and presumably other cerebral petechial hemorrhages, cerebral thrombosis, and brain edema (21). The possibility that hypoxia might initiate angiogenesis in brain and underlie HACE was supported by the finding of increased capillary density in hypoxic rat brain (9, 14, 15) and by the observation that dexamethasone, widely used to prevent and treat HACE, is an effective blocker of angiogenesis (21).

Tissue hypoxia is thought to upregulate a series of local factors that contribute to angiogenesis, the growth of new capillary vessels. A complex cascade of cellular responses, triggered by local hypoxia, increased lactate, and/or redox state, results in capillary basement membrane dissolution and rupture, as well as plasma and red blood cell extravasation. Endothelial cell budding and growth toward the hypoxic region normally follow. In recent years, many putative angiogenic factors have been identified, including vascular endothelial growth factor (VEGF), epidermal growth factor, transforming growth factors-α and -β, tumor angiogenesis factor, angiogenin, tumor necrosis factor-α, acidic and basic fibroblast growth factors, platelet-derived endothelial cell growth factor, and interleukin-8. Among these, VEGF is thought to be the most potent and specific in the basement membrane destruction and leakage. VEGF has been described as a specific in vitro endothelial cell mitogen and as an angiogenic inducer in several in vivo models (1). It is also known as a vascular permeability factor by virtue of its permeability-enhancing effects that, on a molar basis, enhanced the permeability of normal venules and small veins with a potency some 50,000 times that of histamine (20). VEGF has been shown to be upregulated by hypoxia both in vitro and in vivo (17, 24). It has not previously been sought during systemic hypoxia in brain where the blood-brain barrier might exclude such protein cytokines.

Angiogenesis in the brain normally occurs only during growth (19). Endothelial cell proliferation is low in the adult brain. Angiogenesis can occur in brain under pathological conditions such as infarction and tumor growth. VEGF has been detected in brain tumor tissue and was reported to be expressed in rat cerebellum and mouse choroid plexus (3, 16) and after surgical trauma (tumor removal) (21). Cerebral venous thrombosis, a complication of HACE, is consistent with the ability of VEGF to increase von Willebrand factor release (4) and thromboplastin activity (7).

We report here the expression of both VEGF mRNA and VEGF protein in rat brain as a function of time of inhational hypoxic exposure, and we suggest that this may contribute to HACE.

**MATERIALS AND METHODS**

Animal experiments. All studies had prior approval of the committee on Animal Research, University of California, San Francisco. Adult Sprague-Dawley rats (Hilltop Strain, Banpin & Kingman) of either sex (weight 280–300 g) were housed in an aquarium with a plastic cover, kept in normal circadian rhythms (dark at night), and were supplied with food and water. A continuous fresh gas flow was supplied to the chamber, keeping the CO2 concentration below 1%. Ambient O2 concentration was reduced to ~9% initially, and it was reduced progressively over the next hours, in response to activity, to as low as 6%. Generally, the O2 concentration was lowered ~1% every 3 h during the first day until the animals showed little activity and a decreased intake of food and water. Eight rats were housed in the same chamber for each group to ensure equal hypoxic exposures for all animals. The rats were harvested one at a time after eight hypoxic exposures of 0 (control), 3, 6, or 12 h or 1, 2, 3, or 6 days. The procedure was repeated for a total of four runs, each with eight rats. General appearance, activity, response to stimuli, and intake and excretion were recorded daily. O2 and CO2 concentrations were continuously monitored over the 6-day period with use of a PDP 11/44 computer and Perkin Elmer 1100 mass spectrometer. The rats were anesthetized with halothane and then decapitated. Brains were quickly re-
moved, frozen with liquid nitrogen, and stored at ~80°C. Brain edema was not quantified.

Biological and biological reagents. Recombinant human VEGF_165 (rHVEGF_165) (R & D Systems, Minneapolis, MN) was diluted with PBS/BSA (0.3%) to 1 µg/5 ml, aliquoted, and stored at ~80°C. VEGF cDNA (393 bp cloned into pcGEM3 plasmid) was a gift from Dr. Larry Brown (Beth Israel Hospital, Boston, MA). FITC-1-cDNA (458 bp in pGEM3Z) was a gift from Dr. Rubin M. Tuder (Department of Pathology, University of Colorado Health Sciences Center). Mouse β-actin (Ambion, Austin, TX) is a linearized pTRPLE script plasmid containing a 250-bp mouse β-actin gene fragment. 28S ribosome (Ambion) is a linearized pTRPLE script plasmid containing a 115-bp cDNA fragment of the human 28S rRNA gene. Polyclonal rabbit antibody against rhVEGF was purchased from Santa Cruz Biotechnology. Anti-rabbit IgG conjugated with horseradish peroxidase was obtained from Vector Laboratories (Burlingame, CA). The 0.24- to 9.5-kb RNA ladder used in Northern blot analysis was obtained from Gibco-BRL Life Technologies. The rainbow-colored protein molecular weight marker used in Western blot analysis was purchased from Amersham Life Science.

Northern blot hybridization. Total RNA was isolated from 100-mg pieces of cerebral cortex of rat brain by using a single-step method. Tissue was homogenized in 1 ml of RNA STAT-60 (TEL-TEST “B”, Friendswood, TX) by using polytron homogenizer. The total RNA was extracted with chloroform and precipitated with isopropanol followed by a wash with 70% ethanol. The RNA pellet was dissolved in Tris-EDTA buffer (pH 7.5), and the optical density was determined by Shimadzu Recording Spectrophotometer UV-1601. Ten micrograms of total RNA were denatured at 65°C in formamide and bromide-containing loading buffer and were subsequently electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde in 1× MOPS-EDTA-NaOAc buffer. RNA was transferred to nylon membranes (Hybond N, Amersham International PLC, Aylesbury, Bucks, UK) in 20× NaCl-NaH2PO4-EDTA buffer. Blots were cross-linked by ultraviolet irradiation (UV Statalinker, Stratagene), prehybridized in a seal-a-meal bag at 50°C for 1 h in prehybridization solution (1 M NaCl, 1% SDS), and then hybridized at 65°C overnight in hybridization solution (1 M NaCl, 1% dextran sulfate, and 100 µg/ml denatured salmon sperm DNA). The cDNA probes used for hybridization were labeled with [32P]dCTP (New England Nuclear, Boston, MA) to a specific activity of 1–2×10⁹ counts·min⁻¹·µg⁻¹ DNA⁻¹ by using the random dexamer labeling method (Redprime DNA Labeling System, Amersham). Denatured labeled DNA probe was added to the hybridization solution to a final concentration of 1×10⁶ counts·min⁻¹·ml⁻¹. After overnight hybridization, the blots were washed twice in 2× NaCl-NaH2PO4-EDTA buffer with 0.1% SDS at room temperature for 5 min each, and then at 65°C for 5–30 min, and were exposed overnight to X-ray film with intensifying screens at ~80°C. Blots probed for VEGF mRNA were stripped by boiling in 0.1% SDS for 5 min, and the blot was left in the solution until the solution returned to room temperature. All the blots were reprobed for β-actin and 28S antisense RNA probe in a similar manner to permit loading and blotting differences between lanes to be compensated. β-Actin and 28S RNA probes were prepared by T7 RNA polymerase and labeled with [32P]UTP (>2,000 Ci/mmol; New England Nuclear, Boston, MA) with the use of MAXIscript in vitro transcription kits (Ambion). The intensity of the signals was quantified by a scanning densitometer.

Western blot analysis. Samples (100 µg) of rat brain cortex from the same brains used for Northern blot analysis were homogenized thoroughly in 1 ml of lysis buffer (0.01 M Tris·HCl, pH 7.6, 0.1 M NaCl, 0.1 mM dithiothreitol, 0.001 M EDTA, 0.1% Na3, 1 µg/ml leupeptin, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotonin, 1% NP-40). The extracts were centrifuged in a microfuge at 12,000 g for 5 min to remove particides. Total protein was determined by using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Forty micrograms of total protein dissolved in sample buffer containing 2-mercaptoethanol were loaded into each individual lane. Twenty micrograms of 1 µg/ml rhVEGF were used as a positive control. The proteins were then separated by 10% SDS-PAGE at 4°C at 15 mA for 30 min followed by 20 mA for 2–3 h until the blue dye reached the bottom of the gel. The proteins were then transferred onto Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham) with constant current of 200 mA at 4°C for at least 4 h. After transfer, the nitrocellulose blot was blocked overnight with 10% solution of dry milk to prevent nonspecific staining. It was then incubated for 1 h at room temperature with polyclonal antibody against rhVEGF, at a 1:500 dilution, in 10% dry milk in Tris-buffered saline0.1% Tween, with gentle agitation. Subsequently, the filter was rinsed several times and incubated for 1 h at room temperature with anti-rabbit IgG horseradish peroxidase conjugate at a dilution of 1: 20,000 in 1% solution of dry milk. Immunoreactive proteins were detected with use of the ECL Western Blotting Detection System (Amersham). The membrane was exposed to Hyper film ECL (Amersham) at room temperature for 5 min to 2 h.

Statistics. Results are expressed as a ratio of relative intensity of VEGF to β-actin at the corresponding time point. Mean values for total VEGF/β-actin, upper bands of VEGF/β-actin, as well as lower bands of VEGF/β-actin were compared by unpaired Student’s t-test statistical analysis. Results were considered as statistically significant at P < 0.05.

RESULTS

Expression of VEGF mRNA in response to hypoxia in vivo. Figure 1 shows autoradiographs of Northern blots of total RNA from rat brain cortex for one run, with samples after each of the eight test periods. The top panel shows the hybridization signal for VEGF, the middle panel shows the signal for mouse β-actin, and the bottom panel shows the signal for the 28S ribosome. RNA from rat brain exhibited two hybridization signal bands for VEGF at ~3.9 and 4.7 kb. Hybridization patterns with RNA for β-actin were used as an index of the amount of total RNA applied to each lane. 28S ribosome was also used to correct for loading variation of total RNA on each lane. To normalize the data between individual blots, the relative intensities for the hybridization signal (intensity of VEGF mRNA signal divided by intensity of respective β-actin or 28S) are presented in Fig. 2. There are significant (P < 0.05) differences in the relative intensities of both 3.9 and 4.7 kb, as well as the total VEGF mRNA signal between the normal and hypoxic rat brains. No differences were observed between the relative intensities when β-actin was used as reference and those when 28S was used as reference.

After the rats were exposed to hypoxia for as little as 3 h, their VEGF mRNA levels were remarkably increased, reaching a maximum at ~12 h. Brain VEGF mRNA increased about threefold within the first 24 h.
mal effect occurred in 12–24 h. Enhanced VEGF mRNA persisted for at least 6 days.

Induction of VEGF protein production by hypoxia. We performed SDS-PAGE under reducing conditions and Western blot analysis on homogenized whole brain to detect VEGF protein as expected with VEGF mRNA expression. As shown in Fig. 3, one protein band at 23 kDa was detected with use of a polyclonal antibody to human VEGF. VEGF in rat brain was not increased until 12 h of hypoxia, although the VEGF mRNA increased as early as 3 h in hypoxia. A maximum induction of VEGF protein was reached after 2 and 3 days of hypoxia.

**Upregulation of** flt-1 **mRNA during hypoxia.** Northern blot analysis was performed for the mRNA expression of flt-1, one of the two VEGF receptors, in normal and hypoxic rat brain tissue. Figure 4 showed that flt-1 mRNA was induced in rat brain after 3 days of hypoxia but then fell despite constant severe hypoxia.

**DISCUSSION**

Hypoxic exposure of awake rats induced a significant increase in brain VEGF mRNA expression as well as VEGF protein production. Interestingly, VEGF was upregulated primarily during the first few days of continued hypoxia, similar to the time course encountered in the development of symptoms and signs of HACE in humans ascending too rapidly to high altitude. The expression of VEGF mRNA was increased within 3 h of hypoxia, reached a peak at 12–24 h, and then declined. The production of VEGF protein peaked
at 48 h. Similar results were reported by others. In rat heart muscle after coronary arterial ligation, Hishimoto et al. (10) found that VEGF mRNA was evident at 30 min, peaked at 1–2 h, and decreased at 6 h, suggesting that VEGF is an early and transient regulatory factor in response to hypoxia. Tuder et al. (24) reported that VEGF mRNA increased within 2 h of hypoxia in the isolated perfused anoxic lung. VEGF mRNA was also expressed during chronic hypoxia for up to 32 days (24). Previous evidence showed that VEGF was inducible by local (ischemic) hypoxia in brain (23). It was reported that, in brain tumors, VEGF mRNA was strongly expressed in the most ischemic and necrotic areas while not present in the white matter of normal brain where gliomas normally arise (23).

VEGF expression in rat brain tissue after hypoxia suggests a possible role for VEGF in regulating baseline microvascular permeability, perhaps facilitating tissue nutrition and waste removal. Our observations support the thesis that VEGF may act as a mediator in the process of hypoxic cerebral edema, assuming the role of a link between tissue hypoxia and an angiogenic response. LaManna et al. (14) demonstrated capillary budding in superficial cerebral cortex of rats exposed to 1 wk of 0.5 atm in a hypobaric chamber. They also presented evidence that capillary density in rat brain increased after 3 wk of moderate hypoxia (380 Torr) (15). The increased vascularity of the brain in hypobaric hypoxia progresses from an early phase of the microvascular hypertrophy to later microvascular hyperplasia (9). Although our present results could not determine whether increased expression of VEGF led to neovascularization, other studies showed evidence that sustained production of VEGF in hypoxic brain is likely to elicit an angiogenic response (9). First, there is a significant elevation of VEGF gene expression in highly vascularized and edema-associated brain tumor, compared with brain tumors with less neovascularization and little or no edema (2). In extensively vascularized tumors such as gliomas and glioblastomas, the degree of vascularity appears to be related quantitatively to VEGF expression (23). Second, anti-VEGF antibody can block capillary ingrowth into tumors (13). Third, a cause-and-effect relationship between VEGF production and neovascularization was established through the observations of the angiogenic effect of VEGF. Banai et al. (1) showed that, after 4 wk of treatment, the numerical density of intramyocardial distribution vessels was increased by 89% by administration of VEGF into the coronary bed of ischemic myocardium, compared with saline-treated hearts. Their results suggested involvement of VEGF in coronary capillary regrowth in ischemic hearts (10). In regard to HACE, the permeability-enhancing effect of VEGF, which has been found to be 50,000 times more potent than histamine (20), on microvascular vessels may play an important role in the development of cerebral edema under high-altitude exposure. Its stimulation of von Willebrand factor from endothelial cells (4) and induction of thromboplastin activity (7) may be associated with the formation of cerebral thrombosis and petechial hemorrhage in HACE. In our animal model, brain surface vascularity appeared more congested in the hypoxic animals than in controls. Along with the thrombosis and petechial hemorrhage in HACE, brain edema increases interstitial pressure and may lead to compression closure of capillaries, adding local ischemia to tissue hypoxia, which, in return, is a potent inducer of VEGF production (23).

Two VEGF mRNA transcriptions of 3.9 and 4.7 kb were found by Northern blot analysis in rat brain tissue. The signal intensity of both VEGF mRNA transcriptions at 12–24 h after hypoxia was increased up to threefold in hypoxic brain compared with normal brain. The 3.9-kb pattern of hybridization on Northern blot analysis was identical to that observed by others in rats (10, 16). As previously described (11), VEGF may exist in four different homodimeric molecular species because of alternative splicing of mRNA, with each monomer having 121, 165, 189, or 206 amino acids (VEGF121, VEGF165, VEGF189, and VEGF206, respectively). VEGF165 is the most abundant molecular species in all human tissues except placenta, in which VEGF121 is predominant (11). The VEGF isoforms have different properties in vitro, which may determine their function in vivo. The two shorter forms, VEGF121 and VEGF165, are secreted and likely to be available in physiological conditions (11). In contrast, the longer ones, VEGF189 and VEGF206, are bound to heparin-containing proteoglycans in the cell surface or in the basement membrane. The sequence of VEGF189 is the same as that reported for human vascular permeability factor, a protein identified from tumor cell lines on the basis of its ability to induce vascular leakage and protein extravasation (12). Later observations indicate that all four molecular species of VEGF can promote dye extravasation when applied in a guinea pig skin-permeability assay (11). A fifth splice variant, VEGF145, has also been identified from human endometrium and myometrium (6). The bands at 3.9 and 4.7 kb seem to represent VEGF165 and VEGF189, respectively (16). Western blot analysis of rat brain lysates, resolved under reducing conditions with use of a polyclonal antibody, showed one VEGF band at 23 kDa, which is consistent in size to the monomer of VEGF165.

VEGF is said to bind with high affinity to endothelial cells in arteries, veins, and microvessels through its two tyrosine kinase receptors: the fms-like tyrosine kinase receptor (Flt-1) and the tyrosine kinase receptor (KDR) (8, 22). Both Flt-1 and KDR are expressed exclusively in endothelial cells. Flt-1 was found to be upregulated in tumor endothelial cells, although it is not present in the endothelium of normal brain (18). Studies have disclosed that the expression of the Flt-1 and KDR transcripts can be upregulated by hypoxia both in vitro (5) and in vivo (24). In our animal model, the Flt-1 transcript is upregulated in rat brain after 3 days of hypoxia.

Blocking the action of a paracrine mediator that acts on the vasculature may have a significant inhibitory effect on tumor growth. Treatment with VEGF anti-
body has proved to be therapeutic for several highly vascularized and aggressive malignancies (13). Angiogenesis is inhibited by dexamethasone (21). Cultured bovine aortic and pulmonary artery endothelial cell monolayers subjected to hypoxia showed an increase in monolayer permeability that was prevented by addition of the dexamethasone before or during hypoxia (21). Dexamethasone is the most effective known pharmacological treatment of HACE (21). Its mechanism of action on cerebral edema is unknown. This association suggests that blockade of the first stages of angiogenesis may be involved. The relationship between dexamethasone and VEGF remains to be determined. Anti-VEGF therapy may provide a rational approach for dealing with this illness.

In conclusion, our results demonstrate that the transcription of VEGF and the production of VEGF protein in rat brain were upregulated during the first week of hypoxia. Because VEGF is thought to play an important role in angiogenesis, our findings support the hypothesis that the angiogenesis process may be involved in the development of HACE. However, we did not attempt to demonstrate cerebral edema. Direct evidence for a causative relationship between expression of VEGF and brain edema (or petechial hemorrhage) must await additional studies in which the consequences of altered expression of VEGF in brain tissue will be determined. Further study should also include the potential ways to prevent and treat HACE.

We thank Mary Stafford for excellent animal work. We are grateful to Larry Brown, Rubin M. Tuder, and Masabumi Shibuya for their gift of VEGF cDNA and flt-1 cDNA. Many thanks to Dongwei Guo, Jiangfeng Xu, Mary R. Matili, Franklin Ives, and Shanhong Lin for expert technical assistance and to Michael Matthy, Grace Ma, Peter O’Haro, Timothy P. Quinn, and Hui Yuan for helpful suggestions and comments.

This work was supported by the Department of Anesthesia, University of California, San Francisco, CA.

Present address of F. Xu: 552 MRB II, Dept. of Medicine/Hematology, Vanderbilt Univ. School of Medicine, Nashville, TN 37232-6305.

Address for reprint requests: J. W. Severinghaus, Box 0542, UCSF, San Francisco, CA 94143-0542 (E-mail: jws@itsa.ucsf.edu).

Received 31 March 1997; accepted in final form 20 February 1998.

REFERENCES


