Chronic excessive erythrocytosis induces endothelial activation and damage in mouse brain

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Chronic excessive erythrocytosis induces endothelial activation and damage in mouse brain. Am J Physiol Regul Integr Comp Physiol 290: R678–R684, 2006. First published October 27, 2005; doi:10.1152/ajpregu.00246.2005.—Excessive erythrocytosis results in severely increased blood viscosity, which may have significant detrimental effects on endothelial cells and, ultimately, function of the vascular endothelium. Because blood-brain barrier stability is crucial for normal physiological function, we used our previously characterized erythropoietin-overexpressing transgenic (tg6) mouse line (which has a hematocrit of 0.8–0.9) to investigate the effect of excessive erythrocytosis on vessel number, structure, and integrity in vivo. These mice have abnormally high levels of nitric oxide (NO), a potent proinflammatory molecule, suggesting altered vascular permeability and function. In this study, we observed that brain vessel density of tg6 mice was significantly reduced (16%) and vessel diameter was significantly increased (15%) compared with wild-type mice. Although no significant increase in vascular permeability under normoxic or acute hypoxic conditions (8% O2 for 4 h) were detected, electron-microscopic analysis revealed altered morphological characteristics of the tg6 endothelium. Tg6 brain vascular endothelial cells appeared to be activated, with increased luminal protrusions reminiscent of ongoing inflammatory processes. Consistent with this observation, we detected increased levels of intercellular adhesion molecule-1 and von Willebrand factor, markers of endothelial activation and damage, in brain tissue. We propose that chronic excessive erythrocytosis and sustained high hematocrit cause endothelial damage, which may, ultimately, increase susceptibility to vascular disease.

Adequate oxygen delivery is crucially important for central nervous system function, because the brain has a low capacity for anaerobic metabolism. Plasma concentrations of the hematopoietic growth factor erythropoietin (Epo) control the O2-carrying capacity of the blood (12). Epo is produced primarily in the adult kidney and fetal liver and was originally believed to play a role restricted to stimulation of proliferation of early erythroid precursors and differentiation of the erythroid lineage. However, a diverse array of cells have been identified that produce and/or express the Epo receptor, such as endothelial cells (2, 29, 34), smooth muscle cells, and cells of the central nervous system (10, 30). In vitro, Epo has been shown to regulate a variety of neural functions, including calcium flux (3, 25), neurotransmitter synthesis (26), and cell survival (9, 40). Furthermore, Epo has neurotrophic effects (14, 19), can induce an angiogenic phenotype in cultured endothelial cells (34), is a potent angiogenic factor in vivo (18), and has recently been shown to modulate the hypoxic ventilatory response (41). Clinically, lack of Epo leads to anemia, fatigue, and cellular hypoxia; thus recombinant human Epo is frequently used therapeutically to increase red blood cell number and O2 delivery. However, during therapeutic use of Epo, vascular system defects have arisen, stimulating interest in possible negative interactions of Epo with the endothelium (5, 32).

In particular, excessive erythrocytosis results in abnormally high blood viscosity and is often associated with severe clinical complications, such as hypertension and thromboembolism (6). The effects of Epo overexpression and resultant high hematocrit on blood vessel function and structure have not been fully investigated. Clearly, the integrity of blood vessel wall structure in all organs is essential to facilitate efficient blood transport/perfusion and the subsequent oxygenation of all tissue beds. Vascular remodeling to suit local tissue needs may therefore be an important adaptation to excessive erythrocytosis. Cerebral capillaries and, specifically, the endothelial cells that are in direct contact with high blood viscosity may be significantly altered by excessive erythrocytosis and undergo changes in permeability and structure. Such alterations/modifications in cerebral vascular function may compromise blood-brain barrier (BBB) integrity.

There has been no good animal model for study of adaptive mechanisms to excessive erythrocytosis. We have generated a mouse model (designated tg6) that constitutively overexpresses human Epo in an O2-independent manner. Generation and characterization of the line has been previously described (37, 45, 46). In these mice a hematocrit of 0.8–0.9 at 8–9 wk of age increases blood viscosity without altering blood pressure, heart rate, or cardiac output; exercise performance is reduced, and life span is significantly reduced. Endothelial nitric oxide synthase levels and circulating nitric oxide (NO) levels were markedly elevated, leading to increased vasodilatation, which protects these mice from cardiac complications (37).

Using this transgenic model, we have investigated the impact of excessive erythrocytosis, as a result of Epo overexpression, on brain vascular structure and function. We demonstrate that excessive erythropoiesis, i.e., high hematocrit, elevated blood viscosity, and high NO levels known to contribute to increased vascular permeability, is detrimental to the brain vasculature, ultimately culminating in microvascular/endothelial injury.
MATERIALS AND METHODS

All experiments were performed in accordance with Swiss animal protection laws and Zürich University institutional guidelines. The transgenic mouse line was generated by pronuclear injection of the full-length human Epo cDNA driven by the human platelet-derived growth factor (PDGF) B-chain promoter and has been previously described (37). The resulting mouse line, TgN(PDGFBEPO)/321ZhZ (tg6), showed increased plasma and brain levels of Epo (48) and was bred by mating hemizygous males to wild-type C57BL/6 females. Half of the offspring were hemizygous for the transgene, and the other half were wild-type and served as controls. Male and female mice were used at 4–5 mo of age.

Vessel counting and diameter measurements. Mice were anesthetized with 30% O2 and 4% halothane in N2O and then perfused via the abdominal aorta with reduced (2%) halothane anesthesia according to Forssmann et al. (12a). Briefly, initial blood clearance was performed using an isosmolar solution containing 0.5% procaine hydrochloride and 0.025% heparin followed by fixation with a sodium phosphate-buffered formaldehyde-glutaraldehyde solution (each 1.5%) and then a more concentrated solution of 3% formaldehyde-glutaraldehyde containing 0.05% picric acid. After brain removal, small 1-mm cubes of different brain regions were dissected and postfixed in the second fixative overnight. The cubes were embedded in Epon-araldite resin, cut into 0.5-μm sections, and mounted on glass slides. After they were stained with toluidine blue, the sections were observed microscopically (Axioplan, Zeiss, Oberkochen Germany). Capillary density and luminal area of the microvessels were determined with an image-analyzing system (MCID, Imaging Research, St. Catherine, ON, Canada). In each group, ~14,000 capillaries from 4 mice were analyzed.

Electron microscopy. Mice were perfused through the heart with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4, 350 mosM). Tissue blocks were fixed in the same solution for 3 days. The blocks were postfixed in 0.1 M sodium cacodylate (pH 7.4), dehydrated in ethanol, and embedded in Epon 812. Glass knives were used to obtain 1-μm-thick sections, which were stained with toluidine blue and analyzed using a light microscope. From the thick sections, thin (80 to 90 nm) sections were cut, grasped with Formvar-coated copper grips (Fluka Chemie, Buchs, Switzerland), and then double stained with lead citrate and uranyl acetate. Sections were viewed using a Phillips EM400 electron microscope.

Evans blue permeability assay. A 1% solution of Evans blue dye (2 μg/g body wt iv) was injected into mice via the tail vein. After the injection, the mice were placed in a hypoxic (8% O2) chamber for 4 h after a 1-h acclimatization period from 20% to 8% O2 in 10-min steps per 2% O2 decrease. After normoxic or hypoxic exposure, the mice were anesthetized intraperitoneally with ketamine-xylazine and then perfused intracardially with ice-cold PBS. After 15 min of slow perfusion, the organs were dissected, and the dye retained in the tissue was quantitated with a spectrophotometer and calculated as percent of injected dose. Following Evans blue assay, the organs were postfixed in 0.1 M sodium cacodylate (pH 7.4), dehydrated in ethanol, and embedded in Epon 812. Sections were analyzed for occludens (anti-ZO-1, 1:1,000 dilution; Zymed, San Francisco, CA), or anti-intercellular adhesion molecule-1 (ICAM-1) or anti-von Willebrand factor (vWF; both 1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies overnight at 4°C or for 2 h at room temperature. Membranes were washed with PBS containing 0.1% Tween and then incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody. Chemiluminescent detection was carried out, and the membrane was exposed to Kodak autoradiography film. Normalization was performed by reprobing filters with β-actin or Sp1 (1:5,000 dilution; Sigma, St. Louis, MO).

Statistical analysis. Graphical and statistical analyses were performed using Microsoft Excel and Prism software. Values are means ± SD. Statistical significance (P < 0.05%) was calculated using an unpaired t-test of independent samples with equal variance.

RESULTS

Vessel number and diameter are altered in tg6 mice. We investigated the impact of excessive erythrocytosis on vessel distribution in tg6 mice by measuring the total number of vessels and their diameters compared with wild-type normoxic littermates. The results of this study are graphically presented in Fig. 1. We observed a significant decrease (16%, P = 0.0425) in the overall number of vessels in the transgenic mouse brain (13,401 and 14,000 in tg6 and controls, respec-
HIF-1α protein levels are downregulated in hypoxic brain tissue. Because of difficulties in measuring Po2/PCO2 of the arteries and veins in our mice, we investigated the protein levels of HIF-1α after hypoxic exposure as an indirect measure of cellular hypoxic stress (15). Western blotting of nuclear brain extracts showed that, under hypoxic conditions, induction of HIF-1α is significantly reduced (P = 0.015) in tg6 animals compared with wild-type mice (Fig. 2). Furthermore, induction of a well-characterized HIF-1 target gene, vascular endothelial growth factor (VEGF), is also reduced in tg6 mice subjected to hypoxia (data not shown). This finding supports the notion that O2-carrying capacity in these mice is increased and that this increase may even raise the hypoxic tolerance of tg6 mice compared with wild-type animals.

Permeability of brain vessels remain unaltered in tg6 mice. Excessive erythrocytosis causes increased blood cell number and viscosity, which might have an impact on normal vessel function and BBB integrity. To investigate the integrity of brain vasculature of tg6 mice, i.e., alterations in vessel permeability, we used a quantitative Evans blue dye assay, which enabled us to directly compare dye extravasation from vessels into the surrounding parenchyma in transgenic and wild-type mice exposed to normoxic or acute hypoxic conditions. Figure 3 shows no increase in brain permeability in tg6 mice compared with wild-type mice (n = 10). The brain remained extremely dye impermeable, with no variation in the tg6 or wild-type mice under normoxic or hypoxic conditions. Therefore, not only is the brain vasculature impermeable and stable, acute hypoxic insult does not appear to accelerate breakdown of tight junctions or disturb the BBB.

Tight junction proteins are expressed in tg6 brain. Evans blue extravasation studies revealed that, despite changes in vessel number and morphology, permeability of the BBB remained unaffacted by excessive erythrocytosis in tg6 mice, suggesting that they have developed adaptational characteristics that promote normal vessel function. The permeability of vessel walls is determined and maintained by expression levels of tight junction proteins such as occludin and ZO-1. Thus we employed Western blot analysis to monitor expression levels of these tight junction proteins and to ascertain whether they are increased in tg6 compared with wild-type mice. Interestingly, there was no significant difference in overall levels of occludin in tg6 and their wild-type littermates under normoxic or hypoxic conditions (Fig. 4). Similar results were obtained in Western blot experiments with ZO-1 (data not shown). Although no significant upregulation of protein levels was observed, the reduced number of vessels in tg6 mice indicates an increase in the number of tight junctions per vessel, which may contribute to maintenance of normal vascular function in our tg6 mice.

Electron microscopy shows morphological alterations in brain endothelium. To closely investigate any morphological changes in the microvasculature of tg6 mice, we employed electron-microscopic analysis of brain sections and compared them with brain sections from wild-type controls. In Fig. 5, two representative cross- and longitudinal-section images of individual vessels and surrounding parenchyma from wild-type and tg6 mice are shown. A thin basement membrane surrounds the endothelial cells that line the vessel walls and surrounding tissue of control (wild-type) mice. In endothelium from tg6 mice, however, very distinct changes in structure show a thicker basal membrane. The endothelial cells display an activated phenotype containing a greater number of organelles, such as mitochondria and transport vesicles, and have multiple luminal protrusions. Thus tg6 mice exhibit distinct morphological alterations in overall blood vessel structure and endothelial cells that are reminiscent of an inflammatory response.

Endothelial activation/dysfunction markers are upregulated by excessive erythrocytosis. Morphologically, the transgenic endothelium appeared to be activated. To confirm this observation, we performed Western blot analysis for some well-
established endothelial activation markers. A representative Western blot analysis for intercellular adhesion molecule-1 (ICAM-1) on cytoplasmic extracts from transgenic and control mice shows increased expression levels in all tg6 samples (Fig. 6A). Quantification of all Western blots for ICAM-1 and vWF is shown in Fig. 6B. ICAM-1 and vWF were significantly upregulated by approximately twofold ($P < 0.0045$ and $P < 0.0338$, respectively) in tg6 compared with wild-type mice. These data confirm our electron-microscopic results, indicating ongoing endothelial damage in these mice.

**DISCUSSION**

In this study, we have investigated the direct effects of chronic excessive erythrocytosis on brain vascular structure and function with use of a transgenic mouse line in which hypoxia-independent Epo overexpression results in a hematocrit of 0.8–0.9 without alteration of blood pressure, heart rate, or cardiac output. Despite a more than twofold increase in hematocrit levels and blood volume in our mouse model (45), the vascular system appeared to function adequately, such that changes in vessel number, diameter, and morphology did not cause BBB disruption, even after the mice were subjected to an acute hypoxic challenge (8% O$_2$ for 4 h). An increase in tight junction protein expression per vessel presumably contributes to their impermeable state, such that at 4–5 mo of age, the BBB and the microvascular circulation as a whole are adapted to withstand the adverse effects of excessive erythrocytosis.

There are significantly fewer (16%) brain vessels with increased (15%) luminal diameters in tg6 than in wild-type mice. For this reason, we further calculated that these mice should have a 9% reduction in vessel density in the surrounding tissues; therefore, reduced vessel surface area of these mice due to increased oxygenation? Because it has been difficult to measure the PO$_2$/PCO$_2$ of brain arteries and veins in our mice, our assessment is indirect; HIF-1α is induced in response to cellular hypoxic stress (15) and has been shown to play a significant role in proliferation of endothelial cells and capillary formation during hypoxia (43). Protein levels of HIF-1α during hypoxia and its target gene VEGF are reduced in tg6 compared with wild-type controls. This reduced oxidative stress response supports the notion that oxygenation is improved in transgenic mice and may even increase their hypoxic tolerance compared with wild-type mice.

There is substantial evidence that many endothelial functions are sensitive to the presence of reactive oxygen species (ROS) and oxidative stress. Increased ROS levels result in the production of excess oxidants, which overwhelm the scavenging capacity of cellular antioxidant systems; consequently, protective glutathione stores are depleted. Because a direct cross talk between glutathione and ICAM-1 has recently been proposed (20), increased levels of ICAM-1 in tg6 brain imply that antioxidant levels may be decreased. ROS may also reduce...
local production of protective levels of NO or its bioavailability by reacting and scavenging NO to form peroxynitrite. Indeed, oxidative stress has been shown in many experimental paradigms to result in endothelial damage and dysfunction in vivo (1, 22, 35) and may directly damage the tg6 vasculature. Tg6 brain vascular endothelial cells displayed an activated phenotype with increased basement membrane thickness, denoting augmented deposition of extracellular matrix molecules, both features reminiscent of an inflammatory phenotype. Similar to oxidative stress, inflammation is also important in the development and/or progression of vascular disease and is particularly associated with alterations in the expression of vascular and platelet adhesion molecules, levels of coagulation factors, and activity of oxidants and antioxidants (31, 36). In agreement with this, tg6 brain extracts showed increased expression of ICAM-1 and vWF, well-recognized markers of endothelial activation and damage (27), indicative of an active immune response. These reports confirm our previous observations of significantly increased levels of the endothelium-specific cell adhesion molecule VCAM-1 in tg6 mice (48), alluding to continuous activation and damage of the endothelium. ICAM-1, although not specific to endothelial cells, also plays an important role in immune-mediated cell-cell adhesive interaction and intracellular signal transduction pathways. A strong correlation between induction of ICAM-1 and vascular permeability has been shown in various pathologies, including atherosclerosis, ischemia, human immunodeficiency virus, and autoimmune disorders, suggesting that ICAM-1 mediates regulation of BBB function and structure (13). Although our study did not show increased BBB permeability, it is likely that the elevated ICAM-1 levels are an early indication of imminent loss of BBB integrity. The significantly increased level of endothelium-specific vWF detected in mouse brain extracts further indicates vascular injury and endothelial damage (27) in tg6 brain. Furthermore, elevated vWF levels also facilitate increased platelet adhesion and, thus, can exacerbate endothelial damage (8).

Blood flow may also alter endothelial characteristics. Vascular endothelial cells are constantly subjected to shear stress imposed on them by blood flow, and shear stress may be a critical factor affecting gene expression in endothelial cells during inflammation. Data from in vitro (42) and in vivo (49, 50) studies indicate that low or low-oscillating endothelial shear stress induces molecular and vascular responses that may be instrumental in progression of vascular-related diseases (47). Because shear stress is a function of blood viscosity, blood flow velocity, and vessel diameter, it is very difficult to assess this parameter in capillaries of tg6 mice. Nevertheless, we propose that in our mouse model, normal physiological shear stress that is vasculoprotective and fosters quiescence of the endothelium and vascular wall may be compromised partly as a result of significantly increased hematocrit and, therefore, increased blood viscosity (45), thereby contributing to endothelial damage.

Additionally, blood flow may directly affect endothelial function via stimulation of NO release (17, 44), which mediates vascular relaxation in response to vasoactive substances and shear stress, providing antiproliferative and antiplatelet functions by inhibiting vascular smooth muscle cell proliferation, monocyte adhesion, platelet aggregation, and thrombosis (for a review see Ref. 21). Consistent with these observations, previous work showed that tg6 endothelial NO synthase levels are elevated, resulting in increased vascular NO bioavailability and compensatory vascular dilatation mechanisms (37), and enhanced thrombin-induced NO release may prevent platelet aggregation, cell adhesion, and, in turn, thrombus formation in these mice. On the other hand, NO is known to be proinflammatory at high concentrations (16), causing increased vascular permeability in inflamed tissue, generation of radicals, and induction of inflammatory cytokines (4, 33). Therefore, it is possible that although high NO levels in these animals facilitate beneficial endothelial vasodilatation, bursts of NO production may reach cytotoxic levels (16), with detrimental consequences, as seen after middle cerebral artery occlusion (MCAO) in these mice (48). The production of free radicals associated with NO formation might also sequester monocytes and neutrophils (Mac-1+ cells) and result in increased leukocyte counts, as in tg6 mice (48).

Leukocytes, especially polymorphonuclear neutrophils, also have the ability to cause proteolytic and oxidative damage to vessels via NO release. This was shown by antibody-mediated depletion of these cells in vivo, resulting in reduced infarct volumes after MCAO (23). Moreover, ICAM-1 is essential for integrin-mediated leukocyte adhesion to activated endothelium, and ICAM-1-mediated microvascular failure plays a significant role in ischemia-reperfusion injury (23, 24, 51). The upregulation of ICAM-1 in tg6 mice emphasizes this point and could also help explain the previous negative outcome observed in these mice after MCAO. In the absence of injury, however, because leukocytes express functional Epo receptors (38), Epo may directly interact with and reduce the number of leukocytes and slow the inflammatory response as a whole (39).
Although a detrimental effect of high Epo levels on the vasculature cannot be ruled out, clinically Epo is a very well studied and frequently used compound, and all current evidence suggests that this is unlikely to be the case (for a review see Ref. 28). Thus we hypothesize that changes in the tgf6 endothelium are not a direct consequence of chronically elevated Epo levels in these mice but, rather, are due to the detrimental secondary effects of excessive erythropoiesis. The hypothesis that excessive erythropoiesis results in a state of chronic and/or recurrent inflammation and endothelial dysfunction in our mice correlates well with results from clinical studies of polycythemia vera patients, who displayed endothelial dysfunction and perturbation, which might predispose them to arterial disease (11, 31). Thus our data confirm that endothelial damage is indeed associated with polycythemia vera.

In summary, the work presented here demonstrates that chronic excessive erythropoiesis ultimately results in endothelial activation and altered vascular morphological characteristics that are reminiscent of ongoing inflammatory processes. We propose this animal model to study endothelial dysfunction and vascular changes that may play a central role in the development of vascular-related diseases that cause/culminate in high hematocrit levels or are associated with endothelial activation.

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GRANTS

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REFERENCES


4. Beckmann JS, Beckman TW, Chen J, Marshall PA, and Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 87: 1620–1624, 1990.


