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Research Report

Constitutive excessive erythrocytosis causes inflammation and increased vascular permeability in aged mouse brain

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ABSTRACT

Excessive erythrocytosis results in severely increased blood viscosity that may compromise the vascular endothelium. Using our transgenic mouse model of excessive erythrocytosis we previously showed that despite altered brain endothelial cell morphology and an activated vasculature, brain vascular integrity was largely maintained up to 4–5 months of age. We now present data showing that persistent long-term damage of the vascular wall during the later stages of adulthood (9–12 months) results in a chronic detrimental inflammatory phenotype and increased vessel permeability that likely contributes to the reduced life span of our erythropoietin overexpressing transgenic mouse. In aged transgenic animals inflammatory cells were detected in brain tissue and elevated RNA and protein levels of inflammatory markers such as IL-6 and TNFα were observed in both brain tissue and blood plasma. Additionally, increased expression of p53 and other pro-apoptotic markers, as well as decreased Bcl-xL expression in the brain vasculature, indicated ongoing cell death within the vascular compartment. Finally, abnormally elevated vascular permeability in all organs was detected in correlation with decreased expression of the tight junction protein occludin and the adherens junction protein β-catenin in brain. Thus chronic erythrocytosis results in sustained activation of inflammatory pathways, vascular dysfunction and blood–brain barrier disruption.

1. Introduction

Adequate oxygen delivery is crucially important for brain function because of its low capacity for anaerobic metabolism. The hematopoietic growth factor erythropoietin (Epo) circulates in plasma and controls the oxygen carrying capacity of the blood (Fisher, 2003; Velly et al., 2010). Epo is produced primarily in the adult kidney and fetal liver and was originally believed to play a role restricted to stimulation of early erythroid precursors and differentiation of the erythroid lineage. In addition to its well known role in erythropoiesis however a diverse array of cells have now been identified that produce Epo and/or express the Epo receptor including endothelial cells, smooth muscle cells and cells of the central nervous system (Digicaylioglu et al., 1995; Masuda et al., 1994). In vitro Epo has been shown to regulate a variety

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of neural functions such as calcium flux (Korbel et al., 2004) neurotransmitter synthesis and cell survival (Velly et al., 2010; Vogel et al., 2003). Furthermore Epo has neurotrophic effects (Chen et al., 2007; Grimm et al., 2002; Junk et al., 2002), can induce an angiogenic phenotype in cultured endothelial cells, is a potent angiogenic factor in vivo (Ribatti et al., 2003) and enhances ventilation in hypoxic conditions (Soliz et al., 2005).

Clinically, due to its role in erythrocytosis, lack of Epo leads to anemia with fatigue and cellular hypoxia. Thus recombinant human Epo (rhEpo) is a frequently used therapeutic to increase red blood cell number and improve oxygen delivery. Excessive erythrocytosis however results in abnormally high blood viscosity and is often associated with severe clinical complications such as hypertension and thromboembolism (Bertinieri et al., 1998; Ruschitzka et al., 2000). Notably the effects of elevated Epo levels, and thus resultant high hematocrit, on blood vessel function and structure per se has not been fully investigated. The integrity of blood vessel wall structure is essential to facilitate efficient blood transport/perfusion and the efficient oxygenation of all tissue beds and organs. Vascular remodeling to suit local tissue needs may therefore be an important adaptation to excessive erythrocytosis. Indeed endothelial cells that are in direct contact with high blood viscosity, may be altered and undergo changes in permeability and structure. Such alterations/modifications may compromise blood–brain barrier integrity and disturb neuronal homeostasis in the brain.

To study adaptive mechanisms to excessive erythrocytosis we have generated a mouse model (designated tg6) that constitutively over expresses human Epo in an oxygen-independent manner. Generation and characterization of this line showed these mice have a hematocrit of 0.8–0.9 after the first 8–9 weeks without increased blood pressure or altered cardiac output as well as reduced exercise performance and a significantly reduced life span of 9–12 months (Heinicke et al., 2006; Ogunshola et al., 2006; Ruschitzka et al., 2000; Vogel et al., 2003; Wagner et al., 2001). Interestingly both NO mediated relaxation and circulating NO levels are markedly elevated leading to increased vasodilation and protection from cardiac complications (Ruschitzka et al., 2000).

Using this transgenic model we previously investigated the direct effect of Epo and the impact of excessive erythrocytosis on angiogenesis, blood vessel wall structure and integrity in adult mice of 4–5 months of age (Ogunshola et al., 2006). We observed significant activation of the brain vascular endothelium however, surprisingly, blood–brain barrier permeability remained unaffected (Ogunshola et al., 2006). Thus compensatory mechanisms seemed to enable normal vascular function. This follow up work investigates the long term effects of chronic erythrocytosis in aged animals of 9–12 months. Our data shows that both inflammatory and cell death pathways are strongly stimulated in the vascular compartment and likely cause wide-spread damage to the vascular endothelia. Accordingly, all organs display a significant increase in vascular permeability. Thus during the aging process sustained chronic excessive erythrocytosis activates multiple mechanisms that reduce organ function and ultimately contribute to premature death.

2. Results

2.1. *Endothelial activation markers remain upregulated during aging in mice exhibiting excessive erythrocytosis*

Previously we have shown that chronic excessive erythrocytosis results in activation of the brain vasculature in adult tg6 mice. We now investigated the activation status of the brain vasculature in aged tg6 mice of 9–10 months of age using immunohistochemistry for VCAM-1. Tg6 endothelium was found to be strongly VCAM-1 positive indicating continuous activation of the vascular bed (Fig. 1A) compared to wt animals. Inserts show high magnification of the vascular structures that stain positive for VCAM-1. Western blotting and quantification confirmed this result showing a very strong expression of VCAM-1 in brain lysates of tg6 but not wt animals. Notably, ICAM-1 protein levels were also upregulated (Fig. 1B).

2.2. *Inflammatory mediators and cytokines are elevated in aged tg6 mice*

To further ascertain the impact of chronic vascular activation we performed quantitative polymerase chain reaction (qPCR) analysis of mRNA from tg6 and wt brain tissue. We observed a 2–3 fold increase in both IL-1β and TNFα as well as modest but significantly elevated levels of VCAM-1 and iNOS (Fig. 2A). Thus expression of inflammatory cytokines is significantly increased in the transgenic animals. In blood plasma we also observed that circulating levels of IL-6 were more than doubled compared to wt mice (Fig. 2B), further indicating that inflammatory pathways are chronically induced in tg6 animals. We subsequently identified the main source of the systemically increased IL-6 levels as being the kidney, liver and spleen – organs that are known to undergo significant degeneration during the aging of these mice (Fig. 2C, (Heinicke et al., 2006)). Although brain and heart tissue did not express significantly increased levels of IL-6 the systemically increased IL-6 suggested the possibility of increased infiltration of inflammatory cells within many tissues including the brain parenchyma. Accordingly, we detected a limited number of inflammatory cells usually absent from the normal brain in aged tg6 mice (Fig. 2D). Indicative of an impairment of the blood–brain barrier, the infiltrating macrophages ($CD11b^+$) and T-cells ($CD4^+$, data not shown) were frequently detected in or around blood vessels in the transgenic brain.

2.3. *Increased cell death in tg6 brain vasculature*

The inflammatory phenotype observed in aged tg6 mice suggested a significant detrimental effect on the vascular endothelial cells. Accordingly we observed reduced expression of Bcl-2 mRNA (Fig. 2A) and increased protein expression of pro-apoptotic proteins such as p53, Bax and Bnip3 in tg6 brain tissue compared to wt (Fig. 3A). Concomitantly, reduced levels of the anti-apoptotic protein Bcl-xL were also noted in tg6 brain lysates (Fig. 3B) further highlighting a significant increase in cell death pathways in these animals. Immunohistochemistry demonstrated that in the brain p53 is indeed mainly localized to CD31+ve blood vessels (Fig. 3C and D) and