Excessive erythrocytosis in adult mice overexpressing erythropoietin leads to hepatic, renal, neuronal, and muscular degeneration

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Heinicke, Katja, Oliver Baum, Omolara O. Ogunshola, Johannes Vogel, Thomas Stallmach, David P. Wolfer, Stephan Keller, Klaus Weber, Peter D. Wagner, Max Gassmann, and Valentin Djonov. Excessive erythrocytosis in adult mice overexpressing erythropoietin leads to hepatic, renal, neuronal, and muscular degeneration. Am J Physiol Regul Integr Comp Physiol 291: R947–R956, 2006. First published May 11, 2006; doi:10.1152/ajpregu.00152.2006.—To investigate the consequences of inborn excessive erythrocytosis, we made use of our transgenic mouse line (tg6) that constitutively overexpresses erythropoietin (Epo) in a hypoxia-independent manner, thereby reaching hematocrit levels of up to 0.89. We detected expression of human Epo in the brain and, to a lesser extent, in the lung but not in the heart, kidney, or liver of tg6 mice. Although no acute cardiovascular complications are observed, tg6 animals have a reduced lifespan. Decreased swim performance was observed in 5-mo-old tg6 mice. At about 7 mo, several tg6 animals developed spastic contractions of the hindlimbs followed by paralysis. Morphological analysis by light and electron microscopy showed degenerative processes in liver and kidney characterized by increased vascular permeability, chronic progressive inflammation, hemosiderin deposition, and general vasodilatation. Moreover, most of the animals showed severe nerve fiber degeneration of the sciatic nerve, decreased number of neuromuscular junctions, and degeneration of skeletal muscle fibers. Most probably, the developing demyelinating neuropathy resulted in muscular degeneration demonstrated in the extensor digitorum longus muscle. Taken together, chronically increased Epo levels inducing excessive erythrocytosis leads to multiple organ degeneration and reduced life expectancy. This model allows investigation of the impact of excessive erythrocytosis in individuals suffering from polycythemia vera, chronic mountain sickness, or in subjects tempted to abuse Epo by means of gene doping.

chronic mountain sickness; erythropoietin doping; neurodegeneration; neuromuscular junctions; polycythemia; vascular permeability.

RED BLOOD CELLS are essential for oxygen transport from the lung to all tissues. It is well known that a markedly elevated hematocrit level results from increased erythrocytosis as observed in polycythemia vera, paraneoplastic syndromes, chronic mountain sickness, in lowlanders at high altitude, and in subjects after erythropoietin (Epo) abuse. In turn, elevated hematocrit levels lead to high blood viscosity that puts the cardiovascular system at hemodynamic risk that might lead to death (2, 18, 33, 52). However, some individuals can cope with excessive erythrocytosis. For example, some miners in the South American Andes that live and work at high altitude (37) and are exposed to cobalt, an agent known to induce Epo expression, have been reported to develop unusually high hematocrit levels of 0.75–0.91 (16).

Epo primarily stimulates erythropoiesis through proliferation, differentiation, and maturation of erythroid progenitor cells (9). Reduced oxygen supply increases Epo synthesis through instantaneous stabilization of the hypoxia-inducible factor-1α (HIF-1α; see Refs. 15 and 17). Once stabilized, HIF-1α heterodimerizes with its partner HIF-1β, forms the HIF-1 complex, and binds to the HIF-binding site present on the 3′-flanking region of the Epo gene. However, erythrocytosis can also be caused by increased sensitivity of erythroid progenitors to Epo. Subjects that inherited an autosomal dominant form of the Epo receptor have been described earlier (19). A large pedigree has been described in which erythrocytosis reaching levels of up to 0.68 was transmitted in a dominant fashion. No obvious effect on health of the affected individuals was observed. Moreover, one of the family members has won several Olympic gold medals and world championships in endurance sports.

To follow the consequences of excessive erythrocytosis with a suitable in vivo model, we generated a transgenic mouse line termed tg6 that because of oxygen-independent, constitutive overexpression of human Epo cDNA reaches hematocrit values up to 0.89 during the first eight to nine postnatal weeks (36, 46). Transgenic (tg) mice show a 10- to 12-fold elevation of Epo plasma levels. Plasma volume was not altered, whereas blood volume in tg6 mice was nearly doubled compared with wild-type (wt) siblings (45). Unexpectedly, despite the excessive erythrocytosis, resting adult tg mice showed unchanged heart rate and cardiac output and did not develop hypertension (36, 45, 46) or thromboembolism (51). Adaptational mechanisms involve both J enhanced expression of endothelial nitric oxide synthase that, despite concomitant increased endothelin-1 levels (32), results in systemic nitric oxide (NO)-mediated vasodilation and 2 regulated elevation of blood viscosity by increasing erythrocyte flexibility (44). Despite these adaptive mechanisms, life expectancy of tg6 mice was reduced

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to ~7.4 mo when keeping the animals in conventional cages (46). Of note, an independent tg mouse line that, because of the lack of c-Kit exhibits hematopoietic defects causing perinatal death, could be rescued by breeding with our Epo-overexpressing tg6 mice (47, 48).

Considering that excessive erythrocytosis is observed in various human diseases such as polycythemia vera or chronic mountain sickness, and in respect to the future temptation to abuse Epo by means of Epo-gene doping, we made use of our erythrocytotic mouse line tg6 to investigate the impact of drastically increased red blood cell number on various organs.

MATERIALS AND METHODS

The tg6 mice and Epo expression. The tg mice were generated by pronuclear microinjection of the full-length human Epo cDNA driven by the human platelet-derived growth factor (PDGF) B-chain promoter as described (36). The resulting tg mouse line TgN(PDGFBEPO)321ZhB (termed tg6) showed increased Epo levels in plasma and brain (51). Breeding was performed by mating hemizygous males to wt C57BL/6 females. As expected, one-half of the offspring was hemizygous for the transgene while the other one-half was wt and served as controls. Hematocrit was determined by using standard hematological methodology. Male and female animals were used at the age indicated. To identify the organs expressing human Epo cDNA, Epo levels were determined in 3-mo-old mice (n = 5) by RIA, as described earlier (50). All experiments were performed in accordance with the Swiss and United States animal protection laws and institutional guidelines. Our protocol was approved by the Kantonales Veterinaeramt, Zurich and Bern, Switzerland, and Animal Subjects Committee, University of California, San Diego, CA.

Necropsy. Necropsies were performed according to standard laboratory operating procedures using wt (5.8 ± 1.8 mo, n = 7) and tg6 (7.2 ± 2.2 mo, n = 14) mice. Briefly, animals were anesthetized by an intraperitoneal pentobarbitone sodium injection (~372 mg/kg body wt) or by CO₂ inhalation. Once the death of narcosis was found sufficient, the animals were killed by cutting through the axillary blood vessels and subsequent bleeding.

Exercise performance. Swim speed was measured in 5-mo-old mice (n = 9–12) by video tracking while the animals were learning to find a hidden escape platform (1.5 m diameter, 26°C water temperature) as described in detail elsewhere (54). During the first three trials of training, wt and tg6 mice swam until the maximally allowed swim time of 120 s had elapsed. To assess whether the animals were able to maintain a constant swim speed, we divided those trials into six periods of 20 s and calculated the average swim speed for each period.

We used our custom-made analysis software Wintracker version 2.4. (Ref. 54 and www.dpwolfer.ch/wintracker) and based the analysis on raw data from a Noldus Etho Vision Tracking system (version 1.95, sampling frequency 4.2/s, resolution 256 × 256; Wageningen, The Netherlands). To obtain estimates of effective swim speed, periods when the animals were motionless or attempted to climb up the sidewall of the pool were excluded from the analyses.

Light microscopy analysis and immunohistochemistry. After fixation in 10% (wt/vol) phosphate-buffered formaldehyde solution, the tissues were processed, trimmed, embedded in paraffin wax, sectioned at a thickness from 2–4 μm, and stained by hematoxylin and eosin (H & E). The microscopic findings were recorded during histopathological examination and directly entered in the PathData System (Arta-ceseel, Brielle, NJ). Histological changes were described, wherever possible, according to distribution, severity, and morphological character. Severity scores were assigned on a scale from grades 1 to 5.

Immunohistochemistry on paraffin-embedded sections of mouse kidney with an anti-CD31 antibody was performed using a standard protocol as described before (1). Briefly, after pretreatment with trypsin [0.2 mg/ml in Tris-buffered saline (TBS)/CaCl₂; buffer; Difco, Detroit, MI] for 10 min at 37°C, sections were incubated with the mouse anti-CD31, 1:20 (IC/70A, M-0823; Dako, Glostrup, Denmark), diluted in TBS overnight. Sections were exposed to an affinity-purified biotinylated second antibody (anti-mouse EO433, anti-rabbit EO353, diluted 1:200 in TBS; Dako) for 45 min at ambient temperature, treated with the avidin-biotin-horseradish peroxidase complex (P355; Dako), and visualized by exposing sections to 3-amino-9-ethylcarbazole or 3,3-diaminobenzidine (Sigma Chemical, St. Louis, MO) for 12 min.

Electron microscopy analysis. Small pieces were excised from the tissues and then stored in Karnowsky solution [2.5% (vol/vol) glutaraldehyde, 2% (vol/vol) paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4] at 4°C for several days. The samples were extensively washed in cacodylate buffer (0.1 M sodium cacodylate adjusted with HCl to pH 7.4) and postfixed in 1% (wt/vol) osmium tetroxide (buffered with 0.1 M sodium cacodylate adjusted to 370 mosmol/l and pH 7.4) for 2 h. Subsequently, they were washed in maleate buffer (0.05 M maleic acid adjusted with NaOH to pH 5.0), block-stained with uranyl acetate (0.5% wt/vol in maleate buffer), dehydrated in ethanol, and embedded in Epon (Fluka, Buchs, Switzerland) as reported earlier (5). Semithin sections (1 μm thick) were prepared using histodiamant knives subsequently stained with toluidine blue and analyzed using a Leica Leitz DMR light microscope. Representative areas from the sectioned skeletal muscles were further processed for electron microscopy. Ultra-thin sections (80–90 nm in thickness) were obtained with an ultramicrotome (Ultracut; Reichert-Jung, Bensheim, Germany) and floated on 200 mesh grids. They were stained with uranyl acetate and lead citrate using the Leica EM-stain (Leica, Glatbrugg, Switzerland). Visualization was performed with a transmission electron microscope (EM 300; Philips).

Permeability measurements. A 1% solution of Evan’s blue dye (2 μl/mg mouse wt) was injected intravenously in 4- to 5-mo-old mice (n = 9–12) via the tail vein. Four hours were allowed for circulation within the system, and then the mice were anesthesia intraperitoneally with ketamine/xylazine and perfused intracardially with ice-cold PBS. After 15 min of slow perfusion, liver, kidney, and brain were dissected, and the dye retained in the tissue parenchyma was extracted with formamide (5 μl/mg organ wt) for 72 h at room temperature. Absorbance was then measured at 620 nm.

Quantification of skeletal muscle fibers and neuromuscular junctions. For calculation of the proportion of degenerated skeletal muscle fibers, the numbers of damaged H & E-stained skeletal muscle fibers were determined on five cryostat sections from extensor digitorum longus (EDL) muscle of each four wt and tg6 mice (age: 7 mo old) at a magnification of ×20. The ratio between numbers of degenerated vs. normal muscle fibers in the section was then calculated.

Neuromuscular junctions (NMJ) were detected on the same EDL samples described above by acetylcholine receptor (AChR) immunohistochemical analysis. After preparation, EDL muscles were frozen in liquid nitrogen-cooled methylbutane, mounted on cork plates with TissueTek, and stored in closed plastic bags at −70°C until use. Cryostat sections (10 μm) were fixed for 10 min in acetone, air-dried, and equilibrated in TBS, pH 7.4 for 5 min. AChRs were visualized by incubation with FITC-labeled α-bungarotoxin (Sigma, Buchs, Switzerland) diluted 1:100 in TBS, pH 7.4 for 30 min at room temperature. The sections were then counterstained with hematoxylin and covered-slipped in Moviol (Merck, Darmstadt, Germany). For calculation of NMJ density, the numbers of NMJ and skeletal muscle fibers were counted on each of five cryostat sections from four EDL muscles derived from wt and tg6 mice.

Statistical analysis. Data are expressed as means ± SD. Two-way ANOVA statistics for repeated measurements with factors genotype and time were applied to swim performance data. The two-tailed Student’s t-test for unpaired samples was used 1) to compare the mean values and 2) as a post hoc test to evaluate the significance of differences between the groups at the corresponding time points. The level of statistical significance was set at P ≤ 0.05.
RESULTS

Increased Epo protein levels in tg6 mice. Knowing that plasma Epo levels in tg6 mice are increased such that hematocrit levels of up to 0.89 are reached (36, 45, 46) and considering that the human PDGF B-chain promoter used to express the human Epo cDNA drives expression preferentially, but not exclusively, to neuronal cells (38), we sought to identify organs that overexpress the human Epo gene. To this end, Epo levels were measured in different organs obtained from wt and tg6 mice (3 mo old, n = 5). Epo levels increased 26-fold in the tg6 brain and 2.5-fold in the tg6 lung (Fig. 1). Of note, we observed no differences in Epo expression between wt and tg6 animals in heart, liver, and kidney. Visual inspection showed a very red appearance of the snout and paws of tg6 mice caused by the Epo-induced excessive erythrocytosis (Fig. 1).

Macroscopic and histological analysis of tg6 mice. Necropsy of 5-mo-old tg6 mice revealed a generalized vasodilatation as exemplified in the vessels of the thorax (Fig. 2). Note that the internal thoracic vein is congested because of hypervolemia. Moreover, compared with wt mice, the sternum of tg6 mice was enlarged with a thinner corticalis presumably because of the increased erythropoietic activity.

Reduced endurance performance in tg6 mice. To measure exercise performance, 5-mo-old mice (n = 9–12) were subjected to a 120-s swim test. The initial swim velocities of tg6 mice and wt controls showed no statistical difference (Fig. 3). After 60 s, however, swim speed of tg6 animals steadily decreased significantly, indicating premature exhaustion. In contrast, the swim speed detected in wt mice showed little change over this time period. Thus excessive erythrocytosis did not result in increased but decreased exercise performance.

Multiple organ degeneration in tg6 mice. Closer anatomic and histological examination was performed on 7.2 ± 2.2-mo-old tg6 mice and 5.8 ± 1.8-mo-old wt mice. Multiple organ degeneration was observed in many of the tg6 but not the wt mice. As controls, some organs (liver, kidney, skeletal muscle, sciatic nerve) of 5-mo-old tg6 mice were also investigated, showing no signs of degeneration.

In liver, ~45% of tg6 mice displayed minimal (groups of mononuclear cells surrounding single blood vessels) to slight (mononuclear cell groups extending in liver parenchyma) chronic inflammation (Fig. 4, A–C). The inflammation was characterized by lymphoid cell infiltrations/aggregates sur-

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Fig. 1. Erythropoietin (Epo) protein levels in wild-type (wt) and transgenic (tg6) mouse organs. The tg6 mice showed an intensive erythremia of the snout skin. Increased Epo protein expression was observed in tg6 brain and lung compared with wt mice (3 mo old, n = 5). Data are presented as arithmetic mean values ± SD. ***P ≤ 0.0001.

Fig. 2. Necropsy of 5-mo-old tg6 mice with excessive erythrocytosis. The tg6 mice showed dilated vessels and congestion of the veins, e.g., of the thorax (B). The sternum (*) of tg6 mice was expanded with a thinner corticalis (D) because of the increased erythropoietic activity compared with wt mice. Thorax, including rips, sternum and internal thoracic, costal arteries, and veins, were stained by Elastin-van-Gieson, magnification ×40.
rounding blood vessels. Moreover, we observed microgranulomas at minimal (occasionally one focus), slight (several small foci distributed throughout the liver tissue), or moderate (in >50% of visible liver lobules) degrees. In all of these cases, the lesions were accompanied with hemosiderin at a moderate degree. These alterations prompted us to analyze the liver’s vascular permeability. Indeed, functional analyses revealed increased extravasation of intravenously injected Evan’s blue dye in the liver of 5-mo-old tg animals (Fig. 4D).

The kidneys of tg6 mice (Fig. 5) revealed a higher incidence and severity of degenerative processes, especially tubular basophilia, tubular casts, mononuclear cell foci and/or pyelitis and/or interstitial inflammation as well as glomerulosclerosis in single cases. When there was the combination of tubular basophilia and tubular casts with one of the other aforementioned findings, a minimal chronic progressive nephropathy (CPN) was diagnosed in >1/3 of the tg6 mice vs. a single case in wt mice. Furthermore, we regularly observed that the glomerular urinary space was significantly enlarged in tg6 mice. When analyzed at the ultrastructural level, the basement membrane in kidneys of tg6 mice was significantly enlarged and irregularly developed compared with wt controls. In analogy to observed disturbed function in the liver, extravasation of Evan’s blue dye was significantly increased in the kidney of 5-mo-old tg animals (Fig. 4D). Furthermore, monitoring of the urine composition in 7- to 8-mo-old tg6 and wt mice demonstrated significant proteinuria and erythrocyturia in the tg6 mice (data not shown), indicating that the renal ultrafiltration is massively disturbed in the tg strain.

Nerve fiber degeneration was found in the tg sciatic nerve and in the lumbar nerve roots of the spinal cord. Light microscopic investigations showed reduced axonal density, massive axonal swelling, and atypically misshapen myelin profiles in tg6 (Fig. 6C) compared with wt (Fig. 6A) mice. The sciatic nerve fiber degeneration was characterized by the formation of so-called digestion chambers or vacuoles containing detritus or eosinophilic bodies and/or cell remnants, swollen or shrunken axons, and/or dilatation of myelin sheaths in single nerve fibers, and the distribution ranged from focal to multifocal. For electron microscopy analysis, sciatic nerves of three tg6 mice were prepared at the following three different sites: close to the inguinal fold, at the bifurcation site of the tibial and the common peroneal (fibular) nerves, and at distal sites of both nerves close to the hollow of the knee.

Progressive stages of myelin fiber degeneration revealed a typical pathological feature of ongoing degenerative processes:
compare Fig. 6B (wt) with Fig. 6, D–H (tg6). First, affected nerve fibers were characterized by shrunken axoplasm followed by detachment of the inner lamellae of myelin sheaths and periaxonal cap formation. These capping structures and the Schwann cells contained frequent myelin whorls representing closely packed “myelin bubbles” (Fig. 6D). Other structural abnormalities, including changes in the myelin profiles, such as folding, myelin splitting, and disorganization, marked further steps of degeneration. Of note, these alterations were usually accompanied by swollen mitochondria and vacuolar degeneration of the axoplasm (Fig. 6, E and F). The terminal stages were characterized by myelin ovoids with irregular, detached sheaths in combination with axoplasm debris or failure and symptoms of phagocytic activity (Fig. 6, G and H). Several quantitative and qualitative pathological differences between different levels of the sciatic nerve were found. The number of myelinated fibers per area in tg6 mice was dramatically higher in proximal than distal parts (1,356 ± 281 vs. 197 ± 101 mm⁻²), suggesting that the nerve fiber degeneration is more pronounced toward the periphery. Finally, we detected a decreased number of Schwann cells and a high incidence of endoneurial edema in the distal parts of the sciatic nerve.

Paralysis and neuromuscular degeneration in tg6 mice. One of the most striking phenotypical features we observed in 7- to 8-mo-old tg6 mice was a motor disorder characterized by unilateral spasms of the hindlimb that was not observed in wt mice (Fig. 7, A–C). In one-half of the tg6 animals, histopathological analysis revealed myofiber degeneration in skeletal muscle (EDL) consisting of shrunken, eosinophilic, or basophilic fibers with loss of striation and in some cases infiltrated by mononuclear cells (Fig. 7E). Indeed, quantification demonstrated a higher proportion of degenerated skeletal muscle fibers in tg6 than wt mice at the age of 7 mo (Fig. 7F). Furthermore, NMJ density was reduced in skeletal muscles of tg6 mice (Fig. 7G). Obviously, chronically overexpressed Epo leads directly or indirectly (e.g., excessive erythrocytosis) to neuromuscular degeneration and paralysis in tg6 mice.

DISCUSSION

By generating the human Epo-overexpressing mouse line tg6 that reaches hematocrit levels of up to 0.89, we aimed to establish a unique animal model to study the impact of elevated Epo plasma levels and excessive erythrocytosis that occur in patients suffering from polycythemia vera (42), and in highlanders suffering from chronic mountain sickness (52). We observed severe degenerative processes in liver, kidney, neu-
rons, and skeletal muscle that in concert resulted in markedly reduced life expectancy of mice.

We detected expression of human Epo in the brain and, to a lesser extent, in the lung but not in the heart, kidney, or liver of tg6 mice. This observation is in line with a previous report using the same PDGF B-chain promoter to drive expression of another gene of choice (38). Because brain-expressed Epo is unlikely to cross the blood-brain barrier, we assume that expression and secretion of human Epo in the tg lung is responsible for the 12-fold elevated plasma Epo level observed in tg6 mice compared with wt siblings. Our tg mice showed a generalized vasodilatation of larger vessels because of chronically increased NO production (36), most probably caused by increased blood viscosity (44). Transgenic bone marrow and spleen were enlarged because of increased medullar and extramedullar erythropoiesis. We have shown previously that splenectomy reduces but does not normalize the tg hematocrit (45). This observation confirms that both medullar and extramedullar erythropoiesis are enhanced upon chronically elevated Epo plasma levels.

Several features of tg6 mice, including excessive erythrocytosis and splenomegaly, very closely resemble the symptoms observed in patients suffering from polycythemia vera and other primary polycythemias that are characterized by low Epo

Fig. 6. Degenerative processes in the transgenic sciatic nerve. Semithin (A and C) and ultrathin (B, and D–H) analysis of cross-sectioned sciatic nerve was performed in the middle part close to the bifurcation site. A and B: wt mice displayed regular nerve morphology, including normal myelin profiles, typical axoplasm content (A), and compact and regular stratified myelin lamellae (ML) surrounded by Schwann cells (SC) and collagen fibers (Co). C–H: tg6 animals were characterized by different stages of myelinated fiber degeneration. C: some of the fibers demonstrated massive axonal swelling (arrows), and others were deformed, representing atypically misshapen forms (arrowheads). D: preterminal axons were characterized by shrinking axoplasm and detachment of the inner myelin sheaths (arrowheads). The cleavages were filled with myelin membrane whorls (arrows). E and F: further degeneration progress was characterized by invaginations of the myelin sheets and their progressive destruction up to total disorganization of the myelin architecture (★). The electron-dense axoplasm (A) contained multiple bubbles (arrows) and degenerative mitochondria (arrowheads). G: terminal axons were characterized by amorphous axoplasm debris (arrowhead) or axonal loss (arrow). The myelin sheaths were irregular, detached, and building ovoids. Note the lack of normal Schwann cells in F and G. H: phagocytic Schwann cell containing a degenerated axon (★). Bars in A and C, 20 μm; bars in B and D–H, 2 μm.
plasma concentration in contrast to our model (31). Despite having been described over a century ago and showing an incidence of 2–3/100,000, the etiology of polycythemia vera remains unknown, and there is no consensus to the optimal therapy (42). Life expectancy is reduced in polycythemia vera patients, with erythrocytosis being responsible for severe thrombotic events and eventually leading to death (42). Although our tg6 mice will be useful to improve the therapeutic management of excessive erythrocytosis, we did not observe thromboembolic complications, at least not in 3- to 5-mo-old animals (36, 45). One explanation is that, in contrast to polycythemia vera patients who commonly develop excessive erythrocytosis later in life (peak incidence: 50–70 yr), tg6 mice exhibit a NO-based adaptive mechanism as early as 3–5 wk after birth (36, 46). In other words, lifelong exposure to excessive erythrocytosis might prevent thromboembolic complications, whereas suddenly increased hematocrit levels might be life-threatening.

Some reports show that a given elevation in hematocrit levels enhances exercise performance (7). However, Epo-induced excessive erythrocytosis in tg6 mice does not. We observed drastically reduced swimming performance in 5-mo-old tg6 mice that might be explained by rheological/cardiovascular complications and/or by (neuro)muscular dysfunction. We propose that both conditions are involved in decreased performance but that they are separated over time: although hemodynamic complications occur as soon as the hematocrit increases, degenerative processes in nerves and skeletal muscle may develop slowly (see below). Numerous pathological findings such as increased central venous pressure, increased left ventricular systolic and end-diastolic diameter, reduced fractional shortening of the left ventricle (45, 46), and increased pulmonary artery pressure (13, 49) in tg6 mice make it very likely that the cardiovascular system is a major limiting factor. Indeed, the continuous decrease of performance in the swimming test is distinctive rather for exhaustion caused by cardiovascular problems than for neuromuscular defects. Previous human studies have also suggested that an excessive increase in blood viscosity is involved in impaired gas exchange and reduced exercise performance. This has been observed in cases of polycythemia vera (27), high-altitude polycythemia, and chronic mountain sickness (4, 52, 53). Of note, upon either phlebotomy or hemodilution that reduced the hematocrit from excessive to normal (e.g., sea level) values, individuals experienced a stimulation of ventilation and an improved ventilation-perfusion pattern.
Closer morphological analysis by light and electron microscopy revealed severe degenerative processes in liver, kidney, sciatic nerve, and skeletal muscle obtained from 7- to 8-mo-old tg6 mice. The degenerative processes in liver are comparable to those of patients suffering from hemochromatosis, a genetic disorder causing the body to absorb an excessive amount of iron deposited in various organs, mainly the liver (30). In these patients, the inappropriately increased iron loading of the parenchymal cells and the chronic inflammation leads eventually to cirrhosis of the liver (30). Thus tissue degeneration in hemochromatosis develops in response to iron deposition. In contrast, we observed inflamed and necrotic areas in many organs that were not necessarily impregnated by iron, suggesting that the tissue damage occurring in the aged Epo-overexpressing tg6 mice is not caused by the iron overload.

The kidneys of tg6 mice showed a higher incidence and severity of degenerative characteristics. CPN was diagnosed in 40% of the tg6 mice. CPN is a common age-related disease in rodents (12), but the incidence was significantly higher in tg6 compared with wt mice. Hence, a progression toward early onset of CPN may be considered to be a characteristic finding in tg mice. In patients with renal diseases, a vicious cycle of injury and inflammation, resulting in a similar pattern of progressive nephropathy, occurs relatively independent of the type of initial insult. Data from animal models have shown that reduction in nephron mass exposes the remaining nephrons to adaptive hemodynamic changes that are intended to sustain renal function acutely but may be detrimental for longer time periods (35). Other reports suggest that excessive erythrocytosis plays a role in the development of chronic renal failure in patients with preexisting polycythemia vera (40, 41). On the other hand, patients with chronic mountain sickness at high altitude showed reduced kidney plasma flow and increased filtration fraction as hematocrit rose (22, 25). However, despite these marked alterations in kidney dynamics, the tubular function was intact.

Apart from the histologically described organ degeneration, we observed a functionally disturbed endothelium. Excessive erythrocytosis alters normal vascular characteristics as shown by the elevated vascular permeability and/or susceptibility to injury of the tg6 liver and kidney although, interestingly, permeability in brain at 4–5 mo was unaffected (29). Clinical studies have demonstrated that polycythemia vera is associated with endothelial dysfunction (26) and elevated plasma markers of activation/damage of endothelial cells (8) that might represent an increased risk of developing arterial disease. At present, we cannot propose a mechanism for altered permeability nor are we able to provide data on renal and hepatic blood parameters. This is because of the minimal amount of plasma we obtained from animals with a hematocrit up to 0.89.

Hindlimb paralysis, as observed in most of the 7- to 8-mo-old tg mice, encouraged us to analyze the sciatic nerve, to quantify NMJ, and to investigate the morphology of skeletal muscle. We found nerve fiber degeneration in the lumbar nerve roots and markedly increased degeneration in the sciatic nerve in tg6 mice. Generally, radiculoneuropathy is recorded in laboratory rodents in long-term studies at a high frequency as a common background lesion (20). However, in this case, radiculoneuropathy occurred along with an unusually high incidence and severity of fiber degeneration in the sciatic nerve of tg6 compared with wt mice. The motor invalidation, myofiber degeneration, and loss of NMJ in the skeletal muscle of 7-mo-old tg mice are considered to be secondary to the nerve degeneration that may in some cases cause hindlimb paralysis. However, the functional correlation between hindlimb paralysis and sciatic nerve degeneration remains to be determined. Compared with the distal regions, proximal parts of the sciatic nerve were less affected by axonal degeneration and contained a smaller number of myelin profiles and abnormal myelin sheaths. These morphological features resemble those that are characteristic of some inherited demyelinating neuropathies in humans (6). Such chronic disorders of the peripheral nervous system cause muscle weakness and sensory dysfunction. The most common forms are Dejerine-Sottas syndrome, congenital hypomyelination, and Charcot-Marie-Tooth neuropathy (10, 23, 24). Axonal degenerative processes such as muscle atrophy are irreversible and particularly affect the nerves of the lower limbs.

It seems reasonable to assume that a permanent existence of hematocrit-dependent microthromboses inside the nerve might lead to local lesions. Although the adjacent Schwann cells continuously repair these microlesions, they might accumulate, resulting finally in axon degeneration. The longer an axon is, the more probable it is to develop microlesions. This would explain why axonal pathology appears preferentially in the sciatic nerve and increases in the distal direction. The tg mice overexpressing Epo showed local nerve lesions that include reduced axonal density, massive axonal swelling, atypical misshapen myelin profiles, and progressive stages of myelin fiber degeneration. Further studies are needed to determine whether these nerve lesions are the result of microthrombosis accumulation or caused by other (Epo-mediated) mechanisms. Decreases in the capillary density in brain (29) and skeletal muscle (Baum, unpublished results) of tg6 mice compared with wt mice support the hypothesis that the microvascular supply is also reduced in peripheral nerves of tg6 mice.

As mentioned above, skeletal muscle degeneration might be a consequence of degenerating processes of the corresponding nerves and NMJ. There is, however, another possible explanation: Knowing that Epo stimulates proliferation of myoblasts by binding to the Epo receptor present on satellite cells, one might speculate that chronic stimulation of the skeletal muscle’s stem cells has a diametral effect on the adult musculature (21, 28). Indeed, apart from its erythropoietic function (11), Epo has a generalized role, most probably as an anti-apoptotic agent (43). Resulting from a chronic over-stimulation of Epo receptors on satellite cells, this protective, anti-apoptotic function might be disturbed. However, the design of the present study does not allow differentiation between direct or secondary Epo effects (e.g., excessive erythrocytosis).

The abuse of recombinant human Epo (rhEpo) as a performance-enhancing agent is dangerous and sometimes lethal (33, 39). Epo’s effects are the opposite of those of endurance training (14), namely a change in red cell mass without an increase in the total blood volume (7). Until now, the fight against Epo abuse in athletes is basically limited to its detection in blood and urine samples. In the near future, sport agencies unfortunately will be faced with genetically modified subjects, e.g., gene doping with Epo. Our contribution focuses on prevention by providing scientific evidence of the long-term life-threatening risks chronic Epo-abusing athletes expose themselves to. Whereas in athletes using rhEpo the health risk
subsidies when rhEpo is discharged from the body and red cell production returns to normal values, the use of Epo gene doping conceals uncontrolled long-term health risks. In contrast to the life-long adaptation of our tg6 mice, a rapidly developing excessive erythrocytosis is lethal, as shown in wt mice upon application of Epo cDNA (3).

Finally, one might argue that rhEpo has been therapeutically applied to patients with renal disease for nearly two decades without observing Epo-induced degenerative processes despite long-term application of rhEpo. Compared with the hematocrit levels observed in tg6- or in Epo-abusing subjects, however, correction of the hematocrit in end-stage renal disease patients with anemia has been advocated in a range from 0.33 to 0.36 (34). Presumably, low hematocrit levels prevent these patients from the degenerative processes presented in the current work.

In conclusion, chronically increased Epo levels induce excessive erythrocytosis and lead to multiple organ degeneration, providing an explanation for reduced life expectancy. We propose to use this mouse model to further investigate the impact of excessive erythrocytosis.

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REFERENCES


