Brief Communications

Vascular Endothelial Growth Factor Overexpression Delays Neurodegeneration and Prolongs Survival in Amyotrophic Lateral Sclerosis Mice

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We sought genetic evidence for the involvement of neuronal vascular endothelial growth factor (VEGF) in amyotrophic lateral sclerosis (ALS). Mice expressing human ALS mutant superoxide dismutase-1 (SOD1) were crossed with mice that overexpress VEGF in neurons (VEGF$^{+/+}$). We report that SOD1^{G93A}/VEGF$^{+/+}$ double-transgenic mice show delayed motor neuron loss, delayed motor impairment, and prolonged survival compared with SOD1^{G93A} single transgenics. These findings indicate that neuronal VEGF protects against motor neuron degeneration, and may have therapeutic implications for ALS.

Key words: **vascular endothelial growth factor; amyotrophic lateral sclerosis; motor neuron; superoxide dismutase-1; transgenic; neurodegeneration**

Introduction

Amyotrophic lateral sclerosis (ALS), or Lou Gehrig's disease, is a devastating degenerative disease of upper and lower motor neurons that affects ~2 people per 100,000 and produces weakness, muscle atrophy, fasciculations, spasticity, hyporeflexia or hyperreflexia, and extensor plantar responses (Rowland and Shneider, 2001). Existing treatment provides only marginal benefit and death usually occurs within 5 years of onset. In most patients with ALS the cause is unknown, but 5–10% of cases are familial. Patients with familial ALS have been found to have mutations in any of several genes, including Cu/Zn superoxide dismutase-1 (*SOD1*) (Rosen et al., 1993), alsin (Hadano et al., 2001; Yang et al., 2001), senataxin (Chen et al., 2004), vesicle-associated membrane protein B (Nishimura et al., 2004), and angiogenin (Greenway et al., 2006). Expression of ALS-associated human SOD1 mutations (e.g., *SOD1*^{G93A}) in mice produces a dominantly inherited syndrome with clinical and histopathological features of ALS, including limb weakness, muscle atrophy, and loss of motor neurons (Gurney et al., 1994).

Recent studies have implicated vascular endothelial growth factor (VEGF) in ALS, and suggest that it might have therapeutic potential. Mice with deletion of the hypoxia-response element in the VEGF promoter region (VEGF$^{\delta/\delta}$) develop adult-onset progressive motor neuron degeneration reminiscent of ALS (Oosthuyse et al., 2001). Crossbreeding of VEGF$^{\delta/\delta}$ with SOD1^{G93A} mice produces VEGF$^{\delta/\delta}$/SOD1^{G93A} double mutants with more severe weakness and earlier death than mice carrying the SOD1^{G93A} gene alone (Lambrechts et al., 2003). Intraperitoneal (Zheng et al., 2004) or intracerebroventricular (Storkebaum et al., 2005) administration of VEGF, or intramuscular delivery of a VEGF-expressing lentiviral vector (Azzouz et al., 2004), delays the onset of paralysis, improves motor performance, and prolongs survival in SOD1^{G93A} mice or rats. These findings may have direct clinical relevance, because variations in the human VEGF promoter/leader sequence are associated with reduced levels of circulating VEGF, and confer increased risk for ALS in some populations (Lambrechts et al., 2003).

Materials and Methods

Experiments were performed according to National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee. Male mice expressing wild-type human SOD1 [TgN(SOD1)2Gur] and human ALS mutant SOD1 [TgN(SOD1-G93A)1Gur] (Gurney et al., 1994) on a C57BL/6 genetic background were purchased from the Jackson Laboratory (West Grove, PA) and maintained as SOD1^{G93A} hemizygotes. Mice overexpressing human VEGF$_{165}$ [TgN(NSEVEGF)1651–1653] driven by the rat neuron-specific enolase promoter (VEGF$^{+/+}$, strain V1) from the Department of Neurology, University of Zurich (Zurich, Switzerland) were maintain on a C57BL/6 background (Wang et al., 2005). SOD1^{G93A} and VEGF$^{+/+}$ mice were cross-bred and genotyped by PCR of tail-tip DNA, using 5′-AGGAGAGATGAGCTTCCTACAG-3′ and 5′-GATGGCTGGCAACTAGAAGGCAC-3′ as primers for *VEGF*, and 5′-CATCAgCCCTAATCCATCTgA-3′ and 5′-CgCgACTAACAATCAAAgTgA-3′ as primers for *SOD1*. Age-matched male littermates ($n = 6$) were used as controls in all experiments.

Western blots were performed to determine the expression levels of VEGF and mutant SOD1 in the spinal cord of wild-type and transgenic mice using rabbit anti-VEGF antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human SOD1 (#SOD-100; 1:200; Millipore, Temecula, CA), and rabbit anti-mouse/rat SOD1 (#SOD-101;

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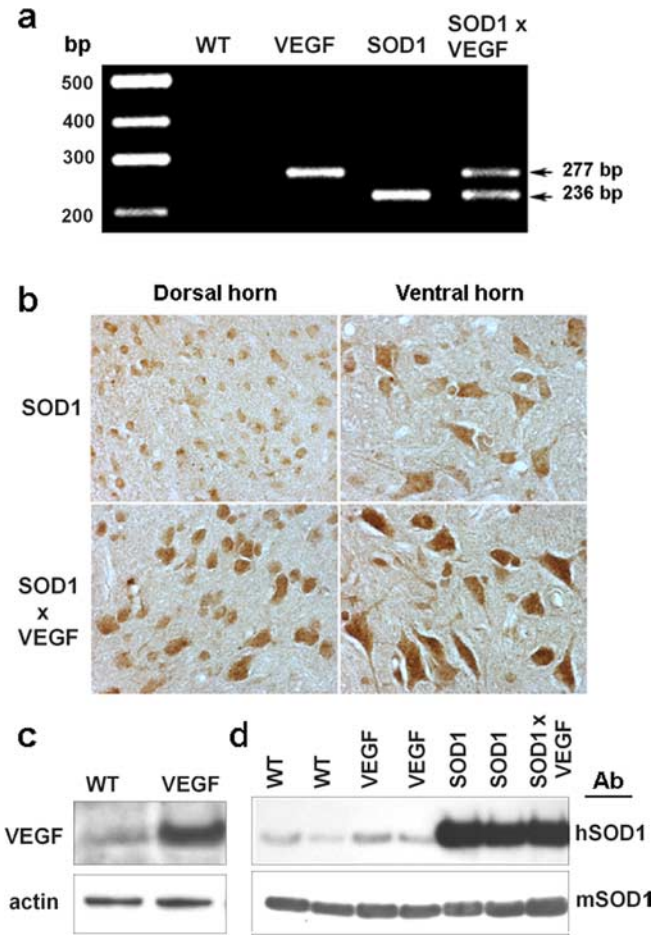

Figure 1. Neuronal overexpression of $hVEGF_{165}$ in $SOD1^{G93A}/VEGF^{+/+}$ mice. ***a***, PCR of mouse-tail genomic DNA shows a 236 bp band corresponding to mutant *hSOD1* in $SOD1^{G93A}$ mice, a 277 bp band representing $hVEGF_{165}$ in $VEGF^{+/+}$ mice, and both bands in $SOD1^{G93A}/VEGF^{+/+}$ double transgenics. ***b***, VEGF immunohistochemistry in lumbar spinal cord shows increased neuronal expression of VEGF (brown) in both ventral and dorsal horns of $VEGF^{+/+}$ mice. ***c***, Western blot confirmed that VEGF was overexpressed in the spinal cord in $VEGF^{+/+}$ mice. ***d***, SOD1 mutant protein was detected in both $SOD1^{G93A}$ and $SOD1^{G93A}/VEGF^{+/+}$ mice, and the $VEGF^{+/+}$ mutation did not affect SOD1 abundance, whether the latter was measured using an anti-human SOD1-preferring (hSOD1, top) or anti-mouse/rat SOD1-preferring (mSOD1, bottom) antibody. Note that anti-hSOD1 detects some mSOD1 (Shinder et al., 2001). WT, Wild-type.

1:200; Millipore) species-preferring antibodies (Shinder et al., 2001), and a standard protocol as described previously (Jin et al., 2000). Antibodies were removed with stripping buffer, and the membranes were reprobed with anti-actin antibody for control of protein loading.

For the rotarod testing, mice were trained for 1 week to perform on an accelerating rotarod (Ugo Basile, Comerio, Italy). Baseline performance was measured at age 60 d, before disease onset, and repeated every 10 d. Each test consisted of three trials, and the best performance (longest duration on the rod without falling, up to a maximum of 180 s) was recorded.

Perfusion-fixed spinal cords were embedded in paraffin, and serial transverse sections (7 μm) through the lumbar spinal cord were cut. Every fifth section was collected, deparaffinized, and stained with cresyl violet. Immunostaining for VEGF and choline acetyltransferase (ChAT) was performed using monoclonal anti-VEGF (1:200; Millipore) and goat polyclonal ant-ChAT (1:500; Millipore), and visualized with diaminobenzidine and hydrogen peroxide.

Quantitative data were expressed as mean ± SEM and ANOVA and Student's *t* test were used for statistical analysis, with $p < 0.05$ considered significant.

Results

If functional VEGF deficiency is an important pathogenic factor for ALS, then overexpression of VEGF should attenuate associated deficits. To test this prediction, we crossed $SOD1^{G93A}$ mutant mice with transgenic ($VEGF^{+/+}$) mice that selectively overexpress $hVEGF_{165}$ in neurons (Wang et al., 2005). PCR of mouse-tail genomic DNA showed a band corresponding to mutant *SOD1* in $SOD1^{G93A}$ mice, and a band representing $hVEGF_{165}$ in $VEGF^{+/+}$ mice, which were absent in wild-type littermates (Fig. 1*a*). VEGF immunohistochemistry and Western blotting demonstrated increased VEGF expression in both ventral horn motor neurons and dorsal horn neurons in the lumbar spinal cord of 60-d-old $SOD1^{G93A}/VEGF^{+/+}$ compared with 60-d-old (presymptomatic) $SOD1^{G93A}$ mice (Fig. 1*b*,*c*). Mutant SOD1 protein was detected in both $SOD1^{G93A}$ and $SOD1^{G93A}/VEGF^{+/+}$ double-transgenics, and the $VEGF^{+/+}$ mutation did not appear to affect the abundance of either the endogenous (murine) or transgenic (mutant human) form of SOD1 (Fig. 1*d*).

In principle, transgenic overexpression of VEGF could mistakenly appear to affect the course of disease in $SOD1^{G93A}$ mice if it acted during development to increase the baseline number of spinal cord motor neurons. To address this possibility, we stained lumbar spinal cord sections from 4-week-old (presymptomatic) adult mice with an antibody against the motor neuron marker

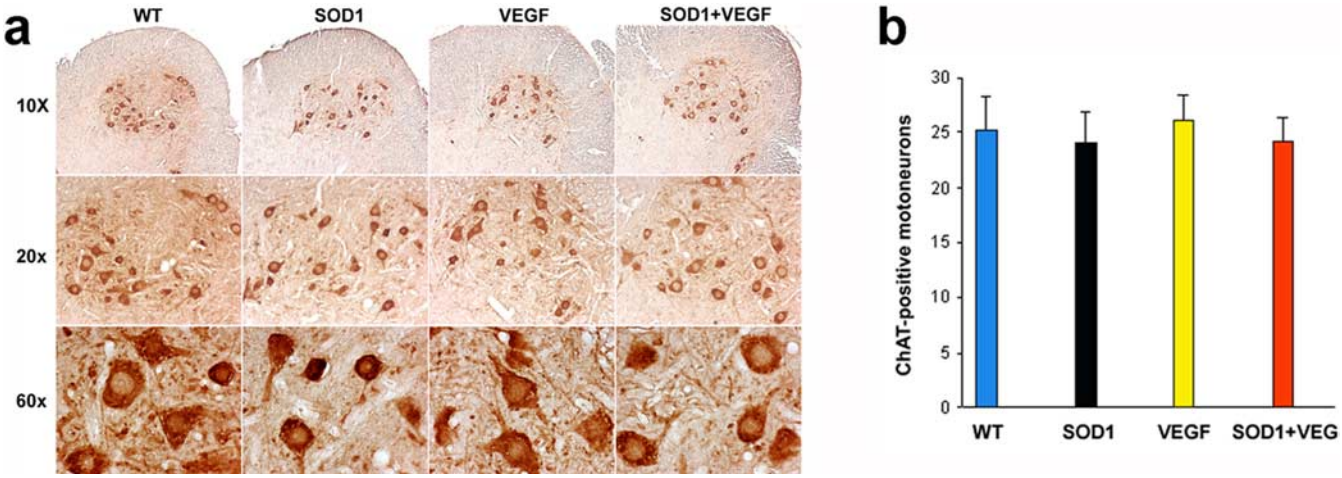

Figure 2. Equivalence of spinal cord motor neuron numbers in 4-week-old wild-type (WT), $VEGF^{+/+}$, $SOD1^{G93A}$, and $SOD1^{G93A}/VEGF^{+/+}$ mice. ***a***, Sections through the ventral horn of the lumbar spinal cord show similar numbers of ChAT-immunopositive cells. ***b***, Cell counts show no differences in ChAT-immunopositive cell counts among the four groups of mice.

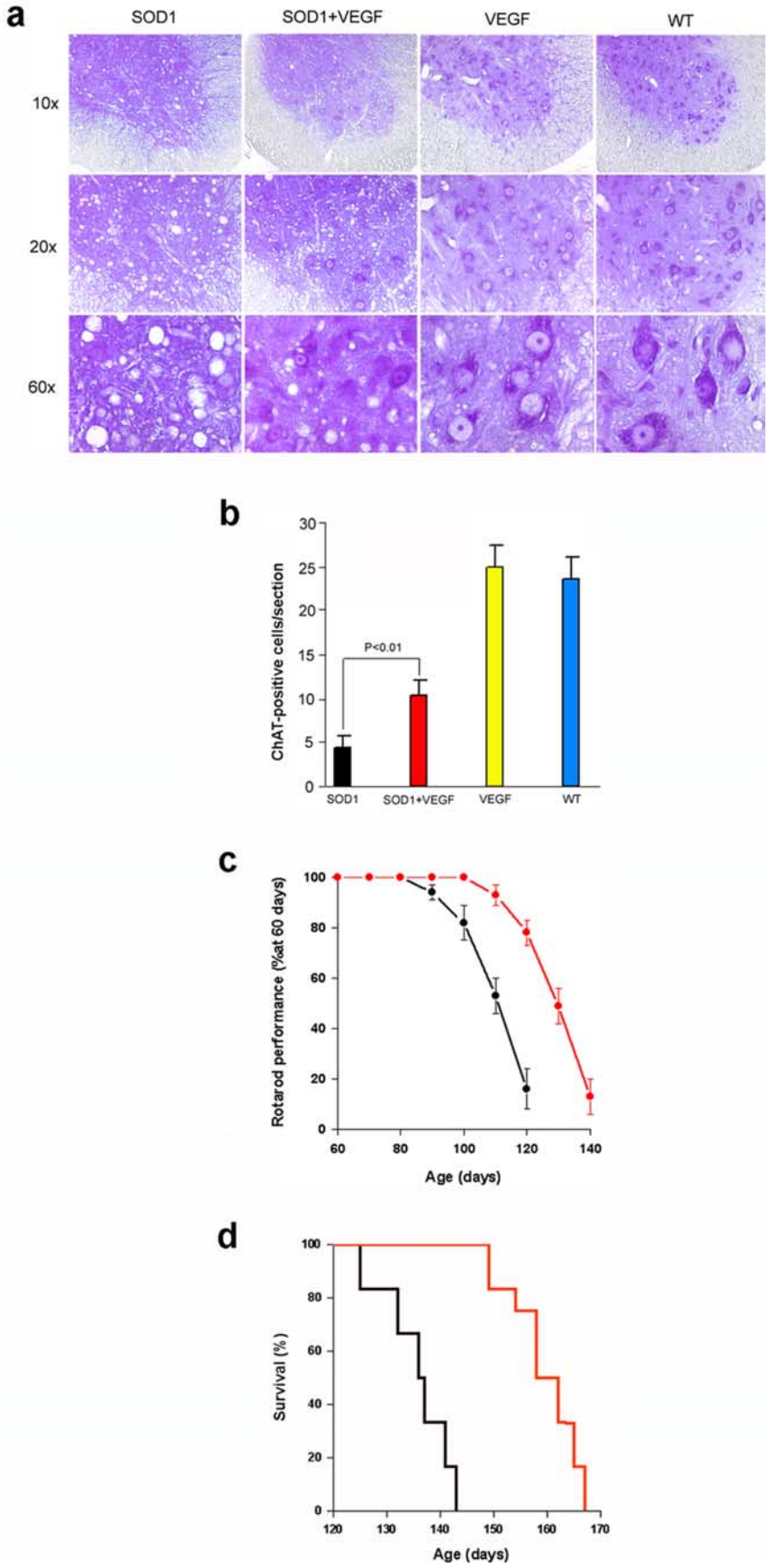

Figure 3. Effect of transgenic overexpression of $hVEGF_{165}$ on the course of disease in ALS transgenic ($SOD1^{G93A}$) mice. ***a***, Cresyl violet-stained sections through the ventral horn of lumbar spinal cord show partial preservation of neurons in $SOD1^{G93A}$/$VEGF^{+/+}$ compared with $SOD1^{G93A}$ mice. ***b***, Survival of cholinergic (ChAT-immunopositive) cells was also increased in $SOD1^{G93A}$/$VEGF^{+/+}$ compared with $SOD1^{G93A}$ mice. ***c***, Impairment of rotarod peformance, measured as latency to fall on an accelerating rotarod and expressed as a percentage of performance at age 60 d ± SEM, was delayed in $SOD1^{G93A}$/$VEGF^{+/+}$ (red) compared with $SOD1^{G93A}$ (black) mice. ***d***, Onset of mortality was delayed, and mean and maximal survival was prolonged in $SOD1^{G93A}$/$VEGF^{+/+}$ (red) compared with $SOD1^{G93A}$ (black) mice. $n = 6$ per group. WT, Wild-type.

ChAT, and counted cells in wild-type, $VEGF^{+/+}$, $SOD1^{G93A}$, and $SOD1^{G93A}$/$VEGF^{+/+}$ mice. However, no differences were observed among these four groups (Fig. 2*a*,*b*).

The most striking histopathological finding in patients with ALS and in $SOD1^{G93A}$ mice is loss of motor neurons in the anterior horns of the spinal cord. Compared with wild-type littermates (or $VEGF^{+/+}$ mice), $SOD1^{G93A}$ mice show prominent depletion of anterior horn motor neurons (Fig. 3*a*,*b*). However, motor-neuron loss was markedly reduced in $SOD1^{G93A}$/$VEGF^{+/+}$ double transgenics. Motor impairment was delayed in onset in $SOD1^{G93A}$/$VEGF^{+/+}$ mice, although its rate of progression, once manifested, was not appreciably altered. Thus, rotarod performance began to decline ~20 d later in $SOD1^{G93A}$/$VEGF^{+/+}$ than in $SOD1^{G93A}$ mice, but deteriorated at the same rate thereafter (Fig. 3*c*). Finally, compared with $SOD1^{G93A}$ mice, $SOD1^{G93A}$/$VEGF^{+/+}$ mice showed a delay in the onset of mortality (149 vs 129 d), and prolonged average (159 ± 3 vs 136 ± 3 d) and maximal (167 vs 143 d) survival (Fig. 3*d*).

Discussion

The ability of transgenically expressed neuronal VEGF to postpone the onset of motor neuron disease in ALS mice is consistent with previous evidence for a protective effect of VEGF in this disorder, and may shed new light on the mechanisms involved. Because VEGF overexpression in our $VEGF^{+/+}$ and $SOD1^{G93A}$/$VEGF^{+/+}$ mice was confined to neurons, neuronal overexpression of VEGF seems sufficient for protection, consistent with the observation that selective neuronal infection with a VEGF-expressing lentiviral vector moderates the course of disease in $SOD1^{G93A}$ mice (Azzouz et al., 2004). Because VEGF is a secreted protein, this does not necessarily imply that motor neurons are the cellular targets of neuronal VEGF in $SOD1^{G93A}$/$VEGF^{+/+}$ mice. However, autocrine (Ogunshola et al., 2002) and direct neuroprotective (Jin et al., 2000) effects of VEGF are well documented, and transgenic neuronal overexpression of the

VEGFR2 receptor also prolongs survival in SOD1^{G93A} mice (Storkebaum et al., 2005), so the mechanism of action of VEGF in this murine model of ALS could be exclusively neuronal.

It is also notable that early onset of VEGF therapy, as achieved in our SOD1^{G93A}/VEGF$^{+/+}$ transgenic mice or in SOD1^{G93A} mice given a VEGF-expressing lentivirus vector beginning at 21 rather than 90 d of age (Azzouz et al., 2004) produces more marked (17–30%) extension of survival than does treatment started at later times (7–8%) (Zheng et al., 2004; Storkebaum et al., 2005). This could relate to differences in the levels of VEGF achieved in these different models, but it could also imply that VEGF induces early adaptations that reduce the sensitivity of SOD1^{G93A} motor neurons to degeneration in advance of its onset.

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