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Vitamin D confers protection to motoneurons and is a prognostic factor of amyotrophic lateral sclerosis

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is an incurable paralytic disorder primarily typified by the selective and progressive degeneration of motoneurons in the brain and spinal cord. ALS causes muscle wasting and atrophy, resulting eventually in respiratory failure and death within 3–5 years of diagnosis. Vitamin D is a potent secosteroid hormone with diverse biological functions that include protection against neuronal damage. The detrimental consequences of vitamin D dietary deficiency have been documented in other neurodegenerative diseases. However, the protective effect of vitamin D on motoneuron and the influence of its levels on disease course remains elusive. Here we found that the biologically active form of vitamin D significantly potentiated the effect of neurotrophic factors and prevented motoneurons from a Fas-induced death, while electrophysiological properties of motoneurons were not affected. In ALS patients, we report that a severe vitamin D deficiency accelerates by 4 times the rate of decline and were associated with a marked shorter life expectancy. Our findings support a neuroprotective function of vitamin D on motoneurons and propose vitamin D as a reliable prognostic factor of ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset neurodegenerative disorder characterized by the progressive degeneration of upper and lower motoneurons. Motoneuron loss causes muscle weakness and atrophy, leading to death in a median time of 3 years (Hardiman et al., 2011). Except for rare inherited cases, ALS etiology remains largely unknown. ALS prognosis is highly variable, ranging from few months to more than 30 years. Some ALS-related conditions have been associated with an increased severity such as age of onset, weight loss, or vital capacity (Chio et al., 2009). The influence of other factors such as depression, smoking, dyslipidemia, or statin treatment remains controversial and, to date, no biological marker, detectable before onset, has been unequivocally linked to ALS onset or progression (Alonso et al., 2010; Armon, 2009; Bowser et al., 2011; Dupuis et al., 2008; Paganoni et al., 2011).

Vitamin D is a steroid hormone, which in its active form forms 1α, 25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), is involved in a wide variety of biological processes including calcium (Ca^{2+}) and bone metabolism, regulation of the immune response, or cancer-related metabolic pathways (Abrams et al., 2013). Interestingly, Vitamin D has been proposed to act as a neuroprotective factor in several neurologic disorders or conditions including Parkinson's disease, multiple sclerosis, cognitive troubles, and neurovascular disorders. Moreover, low plasma levels of vitamin D have been associated with an overall worse prognosis (Balion et al., 2012; Kojima et al., 2012; Mowry et al., 2012; Suzuki et al., 2012). These data are consistent with a neuroprotective activity of vitamin D against neuronal damage, thus prompting the recent proposal of vitamin D as a potential treatment option for ALS (Karam and Scelsa, 2011). However, recent works gave differing results in mice expressing ALS-linked mutated superoxide dismutase-1 (SOD1) mice, a key animal model closely resembling the human pathology. Indeed, while vitamin D intake increased the strength of ALS mice, the lifespan of treated mutant mice was unchanged (Gianforcaro and Hamadeh, 2012; Gianforcaro et al., 2013). In another study, a vitamin D_3-deficient diet delayed disease onset but decreased motor performance of SOD1 mutant mice

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(Solomon et al., 2011). However, recently, vitamin D supplementation in a small cohort of ALS patient suggested beneficial effects on the revised ALS functional rating scale (ALSFRS-R) scores (Karam et al., 2013). This evidence led us to further explore the potential role of vitamin D on motoneurons in vitro and in ALS patients.

Here, we show that vitamin D has significant effect on motoneuron survival by potentiating neurotrophic factor activity and by protecting motoneurons from Fas-induced death. Moreover, when we analyzed the plasma levels of vitamin D in ALS patients, we found that low levels of vitamin D were associated with a worse outcome, defined by the rate of decline (severity score) and survival, compared with patients with normal levels.

2. Methods

2.1. Culture reagents

Glial-derived neurotrophic factor (GDNF) was purchased from Sigma-Aldrich (St. Louis, MO, USA), brain-derived neurotrophic factor (BDNF) from ImmunoTools (Friesoythe, Germany) and ciliary neurotrophic factor (CNTF) from R&D Systems (Minneapolis, MN, USA). Rat monoclonal antibody against vitamin D_3 receptor (9A7 clone), soluble human recombinant FasL set, vitamin D_2, vitamin D_3, and $1,25(OH)_2D_3$ were purchased from Enzo Life Sciences (Farmingdale, NY, USA).

2.2. Motoneuron culture

All animal experiments were done in compliance with the European Community and National directives for the care and use of laboratory animals. *Hb9::GFP* mice (T.M. Jessell's laboratory, New York, NY, USA) were maintained on a C57BL/6 background (Charles Rivers laboratories, Wilmington, MA, USA) (Wichterle et al., 2002). For *Hb9::GFP* motoneuron cultures, transgenic embryos at embryonic day 12.5 were sorted under a fluorescence microscope before dissection of the spinal cord. Motoneurons from spinal cord embryos were isolated as described (Arce et al., 1999) modified by (Aebischer et al., 2011), using iodixanol density gradient centrifugation. Motoneurons are plated on poly-ornithine/laminin-treated wells in the presence (or not when mentioned) of a cocktail of neurotrophic factors (NTFs) (0.1 ng/ml GDNF, 1 ng/ml BDNF, and 10 ng/ml CNTF in supplemented neurobasal medium [Invitrogen, Carlsbad, CA, USA]). Supplemented neurobasal contains 2% (vol/vol) horse serum, 25 mM L-glutamate, 25 mM β-mercaptoethanol, 0.5 mM L-glutamine, and 2% (vol/vol) B-27 supplement (Invitrogen).

2.3. Immunocytochemistry

Hb9::GFP motoneurons were seeded at the density of 5000 cells in polyornithine/laminin–coated 12-mm diameter glass coverslip in the presence of NTFs and maintained at 37 °C, 7.5% CO_2 for 24 hours before being treated with vitamin D_2, vitamin D_3, or $1,25(OH)_2D_3$ (100 nM each) for 8 hours. Neurons were fixed on ice first for 15 minutes with 2% formaldehyde in phosphate-buffered saline (PBS)-neurobasal medium (1:1), then for 15 minutes with 3.7% formaldehyde in PBS. Cells were washed 3 times with PBS and incubated for 1 hour at room temperature in PBS containing 4% BSA, 4% donkey serum, and 0.1% triton-X100. The 9A7 primary antibody was diluted in PBS containing 4% BSA, 4% donkey serum, and 0.1% triton-X100 at concentrations of 5 μg/mL. Immunocomplexes were detected using fluorochrome-conjugated secondary antibodies (AlexaFluor555, Invitrogen).

2.4. Electrophysiology

Electrophysiological recordings in motoneurons were done at 20 °C–22 °C after 6 days in vitro. As we previously reported (Hilaire et al., 2005), for action potential recordings, the bathing solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, and the pH was adjusted to 7.4 with NaOH. Recording pipettes were filled with the following solutions: 140 mM KCl, 10 mM HEPES, 2 mM Mg-ATP, 0.5 mM Na_2-GTP, 0.1 mM EGTA, pH 7.35, adjusted with KOH. For Ca^{2+} current recordings, external NaCl and KCl were replaced with TEA-Cl, pH adjusted with CsOH; internal KCl was replaced with CsCl.

Potentials or currents were recorded with an Axopatch 200B amplifier (Molecular devices, Dipsi Industrie, Chatillon, France). The experimental parameters were controlled with a computer equipped with a Digidata 1300 analogue interface (Molecular devices). We used pClamp software (Clampex 8.02; Molecular devices) for data acquisition and analysis. Signals were filtered at 2 or 5 kHz and sampled at 5 or 10 kHz, respectively. Glass electrodes (3–4 MΩ) were made from capillary glass, using a Narishige puller, and coated with paraffin wax to minimize pipette capacitance. For each experiment, to ensure recordings of motoneurons, green fluorescent *Hb9::GFP* neurons with a large somatic diameter (>25 μm) were selected. Vitamin D_3 and $1,25(OH)_2D_3$ (100 nM each) were added to culture 24 hours before electrophysiological recordings.

2.5. Neurite outgrowth and survival assay

For the analysis of neurite outgrowth, motoneurons were cultured for 24 hours before being treated or not with 100 nM of vitamin D_2, vitamin D_3, and $1,25(OH)_2D_3$. Twenty-four hours later, *Hb9::GFP* neurons were immunostained using primary antibodies directed against the green fluorescent protein (GFP) (TP401; Torrey Pines Biolabs, East Orange, NJ, USA; 1:500). Process outgrowth was analyzed by measuring the length of the longest neurite for GFP-positive motoneuron using the ImageJ software and NeuronJ plugin V10.2 (National Institute of Health, Bethesda, Maryland, USA). In each independent experiment, a minimum number of 40 motoneurons per experimental condition were considered for process analysis. Values were expressed relative to the values in the absence of treatment (none, taken as 100%). For trophic factor deprivation and potentiation, motoneurons were plated at the density of 1200 cells per cm^2 in the presence or not of a cocktail of NTFs, vitamin D_2, vitamin D_3, and $1,25(OH)_2D_3$ at the indicated concentration. After 24 hours of culture surviving neurons were directly counted under fluorescence microscopy. sFasL (100 ng/mL in the presence of 1 μg/mL enhancer antibody), vitamin D_2, vitamin D_3, and $1,25(OH)_2D_3$ (100 nM each) were added to motoneurons that were previously grown for 24 hours in the presence of GDNF, BDNF, and CNTF. Forty-eight hours later, neuron survival was determined by directly counting under a fluorescence microscope.

2.6. Patients

All ALS patients followed in our ALS Center that underwent a blood measure for 25-hydroxy vitamin D levels (i.e., vitamin D_2 + vitamin D_3) between January 2010 and December 2011, were considered for inclusion in the retrospective clinical study. For each of them, the diagnosis of ALS fulfilled the diagnostic criteria (Airlie House/El Escorial revised criteria) of either probable or definite ALS (Traynor et al., 2000). The following clinical data for ALS were available for all the patients: the ALSFRS-R score at the time of vitamin D levels measurement (Kollewe et al., 2008); gender, age of birth, age at vitamin D levels measurement, age at ALS onset, date of ALS onset, site of onset, date of death. ALS onset was defined as the