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Overexpression of human HSP27 protects sensory neurons from diabetes

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SUMMARY

Objectives: To evaluate whether augmenting neuronal protective mechanisms might slow or arrest experimental diabetic peripheral neuropathy (DPN). DPN is one of the most common neurodegenerative disorders and is rising in prevalence. How it targets sensory neurons is uncertain; the disorder is irreversible and untreatable. We explored the intrinsic protective properties of overexpressed human HSP27 on experimental DPN. HSP27 is a small pro-survival heat shock protein that also increases axonal regeneration.
Methods: Experimental diabetes was superimposed on mice overexpressing a human HSP27 transgene and its impact was evaluated on epidermal innervation, behavioral tests of sensation and electrophysiological indices of DPN.
Results: Mice that overexpress human HSP27 in their sensory and motor neurons and that were made diabetic for 6 months by streptozotocin treatment were protected from a range of neuropathic abnormalities, including loss of footpad thermal sensation, mechanical allodynia, loss of epidermal innervation, and slowing of sensory conduction velocity. The protection was selective for sensory neurons in comparison to motor neurons and at 6 months provided better protection in female than male mice. Markers of RAGE-NFκB activation were attenuated by the transgene.
Conclusions: The findings support the idea that diabetic polyneuropathy involves a unique, sensory-centric neurodegenerative process which can be reduced by overexpressing a single gene, an important starting point for new disease-modifying therapeutic approaches.

Diabetic polyneuropathy (DPN) may be among the most common neurodegenerative disorders, involving approximately 50% of all persons with diabetes mellitus, now ranked at 6–10% of the global population and rising in prevalence (Wild et al., 2004; Yang et al., 2010). The mechanisms of DPN remain uncertain and the disorder is currently irreversible. Roles for excessive polyol flux, nerve microangiopathy, impaired growth factor availability and free radical stress, among others, have all been considered (Obrosova, 2009; Tomlinson, 2008; Zochodne, 2007). While controversial, a common thread among these ideas is that there is neuronal targeting by a unique and gradual neurodegenerative cascade that involves sensory neurons early. If correct, an approach that augments intrinsic survival pathways would prevent the range of abnormalities that develop during neuropathy in the peripheral nervous system.

Heat shock proteins (HSPs) are candidates for protecting sensory neurons from neurotoxic insults. As inducible molecular chaperones, they assist in the proper folding and assembly of nascent polypeptides and help to target abnormal proteins for lysosomal degradation (Ohtsuka and Suzuki, 2000). HSPs provide constitutive protection to peripheral neurons, since HSP27 gene mutations, for example, are associated with inherited forms of polyneuropathy (Houlden et al., 2008). An association between the presence of polyneuropathy and circulating HSP27 has also been identified in a large cohort of human subjects with type 1 diabetes mellitus (Gruden et al., 2008). HSP27 expression promotes survival of adult sensory and motor neurons after axonal injury, and its expression is elevated in sensory neurons of diabetic rodent models (Benn et al., 2002; Zochodne et al., 2001). HSP27 (or its mouse analog, HSP25) knockdown or overexpression are respectively associated with attenuated or improved axon regeneration in adult sensory neurons (Lewis et al., 1999; Ma et al., 2011).

We hypothesized that overexpression of a human transgene of HSP27 in mice rendered Type 1 diabetic with streptozotocin (STZ) might protect peripheral neurons from progressive degeneration. The findings identified an interesting form of apparent selective sensory neuron protection over a range of behavioral, electrophysiological, structural and molecular neuropathic abnormalities.

Methods

Human HSP27 transgenic mice and breeding

Mice overexpressing hHSP27 were generated in the Woolf lab by microinjecting the prepared transgenic construct into fertilized B6C3F1

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mouse oocytes which were subsequently bred with C57BL/6 mice to establish the transgenic line as recently described with overexpression in both motor and sensory neurons (Ma et al., 2011). In this transgenic line hHSP27 is overexpressed under a neuronal specific promoter (Thy1.2). hHSP27 mice were transferred to the University of Calgary for breeding and diabetes induction. Heterozygous hHSP27 male mice were bred with C57B6J/L female mice.

The protocol was reviewed and approved by the Animal Care Committee, University of Calgary. All groups studied included male and female mice to render gender balance among the study groups.

Prior to assignment of mice to experimental groups at the University of Calgary, mice underwent genotyping to confirm expression of the transgene. For genotyping DNA extraction was performed on ear notch samples using quick gDNA extraction buffer (Sakurai et al., 2008) adding 300 μl of buffer mix to each sample and incubating at 56 °C for 2 hrs. After tissues were dissolved they were incubated at 95–98 °C for 15 min to inactivate the proteinase K enzyme. The samples were spun at 12000 rpm and 200 μl of supernatant was collected. 3 μl of this was used as template for the PCR using a standard protocol for the ABI7000 sequence amplifier and SybrGreen as amplification marker. The standard dissociation protocol shows a clear single peak of product amplification for the each duplicate sample containing the transgene. An internal control gene was used to test gDNA quality. The sequence of primers was follows:

hHsp27 F5 5′-GTCCCTGGATGTCAACCACT-3′
Thy1.2 R4 5′-GGGTTCCTTAGGAGGGACAA-3′
TRCD F 5′-CAAATGTTGCTTGTCTGGTG-3′
TRCD R 5′-GTCAGTCGAGTGCACAGTTT-3′

While not quantitated, we confirmed hHSP27 overexpression at the 6 month endpoint (data not shown) by immunohistochemistry. While protein–protein interactions of the overexpressed transgene may have changed over the 6 month life span of these mice, this work and previous studies have not noted loss of overexpression (Ma et al., 2011). hHSP27 overexpressing transgenic mice and littermate mice received intraperitoneal injections of STZ with citrate buffer vehicle or citrate buffer alone at 2 months of age as previously described. Three consecutive daily doses of 55, 70 and 85 mg/kg STZ in citrate buffer vehicle or citrate buffer vehicle alone (pH = 4.8) were administered. Subsequent fasting tail blood samples were obtained to determine diabetic status. Diabetic status was defined as blood glucose greater than 16.0 mmol/L following an 8-hr fast. STZ injected mice not demonstrating diabetic range fasting glucose measurements were excluded from the study. Fasting blood glucose was re-assessed at 3 and 6 months following induction of diabetes to confirm persistence of diabetes. Glycated hemoglobin was measured at time of harvesting. Glycated hemoglobin testing was performed at Calgary Laboratory Services (Calgary, AB).

Four groups of mice were established through genotyping for presence of the hHSP27 transgene and presence of diabetes: 1. Littermate control group (no transgene, no diabetes); 2. Littermate diabetic group (no transgene, diabetes present); 3. Transgenic control group (transgene present, no diabetes); 4. Transgenic diabetic group (transgene present, diabetes present). Results were given in gender balanced (similar numbers of males and females) in each experimental group. Later subgroup analysis was conducted to compare the impact of gender on some of the endpoints (Supplemental data).

Electrophysiology, behavioral testing

Electrophysiology was performed in mice anesthetized with isofluorane (99.9%; Halocarbon Products Corporation, River Edge, New Jersey) as previously described (Toth et al., 2008) with near nerve temperature at 37.0 °C. Thermal algesia was tested by hindpaw withdrawal latencies to a light heat source (Hargreaves apparatus) and mechanical allodynia by esthesiometer testing (force threshold to induce hindpaw withdrawal) (Calcutt, 2004; Toth et al., 2010; Truong et al., 2003). For mechanical sensitivity, 12 stimulations were averaged for each of the 3 and 6 month timepoints consisting of 2 tests daily per hindpaw for three days. For thermal sensitivity, 6 stimulations were averaged at each of the 3 and 6 month timepoints that consisted of one test per hindpaw daily for three days. Single averaged values for both mechanical and thermal sensitivity at each time point were used for each mouse in subsequent statistical analysis.

Footpad immunohistochemistry

Bilateral hind footpad samples were excised at the time of harvesting. Samples were placed in 2% PLP (paraformaldehyde, lysine and periodate) fixative as previously described. Samples were cryoprotected overnight in 20% glycerol/20% 0.4 M Sorrenson buffer at 4 °C. Samples were then cut in series at 25 μM intervals with a freezing microtome (Polydefkis et al., 2004). Samples were then washed with PBS (1×), 1% Triton × 100/1× PBS then 10% Goat Serum. Samples were stained with PGP9.5, 1:1000 (Rabbit polyclonal); (Encor Biotechnology Inc., Gainesville, FL), washed with PBS 1× then stained with CY3:1:100 (Goat anti-rabbit); (Thermo Fisher Scientific Inc., Rockford, IL). Images were captured using an Olympus laser scanning confocal microscope equipped with epifluorescence at 100× magnification 512 × 512 resolution with a scanning step size of 1 μm. For each mouse, six footpad sections were made and 5 adjacent fields examined for each section. Mean counts were arrived at from 30 individual images/mouse. Areas of the epidermis analyzed were measured (mm²) for each field using Image J software and axon densities calculated.

Dorsal root ganglion immunohistochemistry

Slides were rinsed 3× with PBS for 5 min. Blocking was performed with 1% Normal goat serum, 0.3% Triton-X in PBS for 30 min at room temperature. Antibodies applied were: NFκb p-50 (H-119), rabbit polyclonal Ig/G from Santa Cruz Biotechnology at the dilution of 1:200, RAGE (ab3611) rabbit polyclonal to RAGE from Abcam (Dilution 1:800), activated Caspase-3 (1:100; Anti-cleaved Caspase-3 Rabbit Polyclonal IgG; Trevigen Inc., Gaithersburg, MD) with Alexa Flour 488 Goat anti-rabbit IgG as secondary antibody from Invitrogen (secondary antibody 1:200). Antibodies were applied and left overnight. In the morning, slides were rinsed 3× with PBS for 5 min. Secondary antibody was applied at 1:200 (Alexa Flour 488 Goat anti-rabbit IgG (Invitrogen, Carlsbad, CA)) and then rinsed with 3× PBS for 5 min.

Image analysis was performed using Adobe Photoshop CS4 (Adobe Systems Incorporated, San Jose, CA). To better characterize direct neuronal expression we divided neurons into categories of arbitrary intensity levels: >70 (arbitrary units) were considered positive, medium (70–100), strong (100–150) and very strong (>150). We counted the total number of neurons, number of positive or negative neurons, number of neurons with cytoplasmic staining, number of neurons with nuclear staining. For counting, each mouse had L4 or L5 lumbar ganglia sectioned and counting included 8–12 individual sections (analyzing approximate 250–450 neurons/mouse). This analysis was limited to control nontransgenic, diabetic nontransgenic and diabetic transgenic groups only.

Analysis

Values were calculated as mean ± sem (standard error of mean). One-way analysis of variance (ANOVA) was used to compare mean values between groups with a priori pairwise comparisons between all groups using Tukey's HSD method.