Insulinlike Growth Factor (IGF)-1 Administration Ameliorates Disease Manifestations in a Mouse Model of Spinal and Bulbar Muscular Atrophy

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Spinal and bulbar muscular atrophy is an X-linked motor neuron disease caused by polyglutamine expansion in the androgen receptor. Patients develop slowly progressive proximal muscle weakness, muscle atrophy and fasciculations. Affected individuals often show gynecomastia, testicular atrophy and reduced fertility as a result of mild androgen insensitivity. No effective disease-modifying therapy is currently available for this disease. Our recent studies have demonstrated that insulinlike growth factor (IGF)-1 reduces the mutant androgen receptor toxicity through activation of Akt in vitro, and spinal and bulbar muscular atrophy transgenic mice that also overexpress a noncirculating muscle isoform of IGF-1 have a less severe phenotype. Here we sought to establish the efficacy of daily intraperitoneal injections of mecasermin rinfabate, recombinant human IGF-1 and IGF-1 binding protein 3, in a transgenic mouse model expressing the mutant androgen receptor with an expanded 97 glutamine tract. The study was done in a controlled, randomized, blinded fashion, and, to reflect the clinical settings, the injections were started after the onset of disease manifestations. The treatment resulted in increased Akt phosphorylation and reduced mutant androgen receptor aggregation in muscle. In comparison to vehicle-treated controls, IGF-1–treated transgenic mice showed improved motor performance, attenuated weight loss and increased survival. Our results suggest that peripheral tissue can be targeted to improve the spinal and bulbar muscular atrophy phenotype and indicate that IGF-1 warrants further investigation in clinical trials as a potential treatment for this disease.
that of the disease protein, including phospho-
fected by posttranslational modifications
polyglutamine-expanded proteins is af-
fect the human disease (16,17). Toxicity of
expanded AR protein more accurately re-
role in the toxicity. In agreement with this,
other than the polyglutamine tract play a
protein context is also important in polyg-
other polyglutamine diseases.
modifying therapy for SBMA, or for any
ance with the National Institutes of
Animals and Drug Treatment
MATERIALS AND METHODS
IGF-1 TREATMENT IMPROVES THE PHENOTYPE IN SBMA MICE
that in vitro insulinlike growth factor
IGF-1 activates the phosphatidylinositol
3-kinase–Akt signaling and increases AR
phosphorylation at the Akt consensus site,
resulting in reduced toxicity of the mutant
AR (21), and that SBMA mice genetically
overexpressing a noncircularizing muscle-
specific isoform of IGF-1 have a less se-
vere phenotype (22). Akt, also known as
protein kinase B, is a serine/threonine-
specific protein kinase that plays a key
role in multiple cellular processes.
In this study, we explored the efficacy
of parenteral injections of mecamaserin
rinfabate, recombinant human IGF-1 and
IGF-1 binding protein 3 (rhIGF-1 / IGFBP3), in a transgenic mouse model of
SBMA. To have clinically meaningful re-
results, we designed the study in a con-
trolled, randomized, blinded fashion,
and the treatment was started only after
the onset of disease manifestations.
We found that administration of rhIGF-
1/IGFBP3 increased activation of Akt and
reduced mutant AR aggregation in skele-
tal muscle. Moreover, it improved motor
function and pathology and prolonged
the lifespan of SBMA mice. Our study in-
dicates that IGF-1 mimetics may be a
promising therapeutic strategy for SBMA.

MATERIALS AND METHODS
Animals and Drug Treatment
The study was carried out in accor-
dance with the National Institutes of
Health Guide for the Care and Use of Labora-
tory Animals (23) and was approved by
the National Institute of Neurological Dis-
orders and Stroke (NINDS) Animal Care
Committee. All the experiments were per-
formed in male mice in the F1 generation
derived by crossing C57Bl6 mice with
BDF1 mice. The transgenic mice ex-
pressed mutant AR with a 97 glutamine
repeat (AR97Q) (11). The mice were geno-
typed by polymerase chain reaction (PCR)
with tail DNA as previously described
(11), by using a REDExtract-N-Amp Tis-
sue PCR kit (Sigma, St. Louis, MO, USA)
according to the manufacturer’s instruc-
tions. Recombinant human IGF-1 in com-
plex with human IGFBP-3 (Iplex,
mecasermin rinfabate) was provided by
Insmed Inc. (Monmouth Junction, NJ,
USA). The IGF-1 complex was dissolved
in a stock solution at a concentration of
60 mg/mL in 50 mmol/L sodium acetate
and 105 mmol/L sodium chloride
(pH 5.5). A vehicle solution was used as
the control. The IGF-1 solution and vehi-
cle were stored in aliquots at ~80°C until
use, and once thawed, the vials were kept
at 4°C for a maximum of 24 h. IGF-1
(15 mg/kg/day) or vehicle was injected
intraperitoneally into mice daily starting
at 10 wks of age and continuing for 6 or
10 wks, as indicated in the Results section.
The daily injections and all the analyses
were performed by blinded investigators.

Behavioral and Survival Analysis
Body weight assessment and the
hanging wire test were conducted every
week for IGF-1–treated and vehicle-
treated mice. For the hanging wire test,
the mouse was placed on top of a wire
cage lid. The lid was shaken slightly
three times to cause the mouse to grip
the wires and then the lid was turned
upside down approximately 20 cm
above the cage floor. The latency to fall
was recorded for a maximum time of
60 s (24). We assessed the gait analysis
using the Gait Analysis Treadmill
(Columbus Instruments) and TreadScan
software (CleverSys). The treadmill con-
sisted of a motor-driven transparent
treadmill belt with an angled mirror
mounted below. A high-speed digital
video camera was mounted to record a
ventral view of the mouse on the tread-
mill belt reflected off the mirror; digital
video images were recorded at 100
frames per second. For each 20-s session,
the video was previewed to determine a
minimum of six consecutive step cycles
of consistent walking for video analysis.
For survival analysis, mice were ob-
served daily and sacrificed when the
mice had lost more than 30% of body
weight or showed inability to move or
signs of dehydration.

Biochemical Analysis
The 16-wk-old mice were anesthetized
with isoflurane and sacrificed. Quadriceps
muscles and spinal cord were dis-
sected and snap frozen in liquid nitrogen.
By using a polytron homogenizer, tissue
samples were processed in ice-cold ho-
rogenization buffer (150 mmol/L NaCl,
50 mmol/L Tris, 2 mmol/L EDTA, 1%
sodium deoxycholate, 0.5% Triton X-100,
0.1% sodium dodecyl sulfate [SDS]) con-
taining protease inhibitor and phos-
phatase inhibitor cocktails (Roche). Ho-
mogenates were sonicated and
pre-cleared at 4,000g for 10 min at 4°C.
The soluble fraction was collected and
protein concentrations were determined
using the Bradford reagent (Bio-Rad). Pro-
tein lysates were separated on Tris-glycine
gels, transferred to polyvinylidene fluo-
ride membranes (both Invitrogen) and
probed with the following antibodies: AR
H-280 (sc-13062; Santa Cruz Biotechnol-
ogy, Santa Cruz, CA, USA), phospho-Akt
and total Akt (9271 and 9272, respectively;
Cell Signaling), choline acetyltransferase
(AB144P; Chemicon) and α-tubulin as a
loading control (T6199; Sigma). Western
blots were visualized with peroxidase-
linked secondary antibodies (R&D Sys-
tems) by chemiluminescence detection
(PerkinElmer Life Sciences). Band densi-
ties were quantified by densitometric
analysis by using ImageJ software and
normalized to the α-tubulin values of the
respective samples. Phospho-Akt values
were normalized to band intensities of
both total Akt and α-tubulin.
Taqman Quantitative PCR Analysis

Total RNA was extracted from frozen quadriceps muscle by using Trizol (Invitrogen) as previously described (25). RNA (1 μg) was reverse-transcribed using the cDNA Archive Kit (Applied Biosystems) following the manufacturer’s instructions. Gene expression was measured by quantitative real-time PCR by using an ABI 9900 Sequence Detector System (Applied Biosystems). Specific assays for myogenin (Mm_00446194-m1), acetylcholine receptor α (Chrna1) (Mm_00431629-m1), myogenic differentiation 1 (myoD) (Mm_00440387_m1) and phosphoglycerate kinase 1 (PGK1) (Mm_00435617-m1) were from Applied Biosystems. The level of each transcript was measured with the threshold cycle (Ct) method by using PGK1 mRNA as an endogenous control. The values were normalized to the mean of the wild-type animals in each group, which was assigned as 1 unless otherwise indicated.

Histological Analysis

Quadriceps muscles from 16-wk-old mice were snap frozen in isopentane. Sections of unfixed muscle tissue were cut at 6–8 μm in a –20°C cryostat and processed for hematoxylin and eosin or nicotinamide adenine dinucleotide staining. For immunofluorescence analysis, sections were fixed with 4% paraformaldehyde (PFA) and incubated overnight at 4°C with rabbit anti-laminin (1:80; Sigma). Digital images were captured using a Zeiss Axiovert 100M microscope and analyzed with NIS Elements software for total cross-sectional area (original magnification 10x), total myofiber number (original magnification 10x), myofiber diameter (original magnification 20x) and minimal Feret diameter (original magnification 20x). Tissue sections (8 μm thick) were fixed in 4% PFA, stained with mouse 1C2 antibody (1:20,000; Chemicon) and counterstained with Mayer hematoxylin. For muscle, >500 fibers were counted in randomly selected areas of individual mice. For motor neuron count, spinal cords from anesthetized mice were collected and postfixed for 12 h in 4% PFA. Paraffin-embedded spinal cords were serially sectioned at 6-μm steps, mounted on slides and processed for Nissl staining. Images of 10 contiguous sections, 100 μm apart (original magnification 10x), were analyzed. Motor neurons were identified as cells positive for Nissl staining, with clear nucleus and nucleolus, and a maximum diameter greater than 25 μm. Counting was performed in a blinded fashion.

Statistical Analysis

A two-way mixed-design analysis of variance (ANOVA) was done to compare the effects of genotype on hanging wire performance and body weight over time. Genotype was used as a between-subject
factor, and time was used as a within-subject factor. A log-rank test was used to compare Kaplan-Meier survival between treated versus nontreated AR97Q mice. A two-tailed Student t test or, for data not normally distributed, the Mann-Whitney U test was performed to compare differences between two groups. The data were analyzed using SPSS version 18 software, and \( p \leq 0.05 \) was considered significant.

All supplementary materials are available online at www.molmed.org.

RESULTS

IGF-1 Administration Activates Akt and Reduces Mutant AR Aggregation in Muscle

To investigate whether systemic IGF-1 treatment is effective in SBMA mice, we used mecasermin rinfabate (rhIGF-1/IGFBP3). The circulating half-life of this compound after subcutaneous injection is longer than for IGF-1 alone, and adverse effects related to peak activity, mainly hypoglycemia, may be reduced (26). It has been used in previous clinical trials and is approved by the U.S. Food and Drug Administration for treatment of primary IGF-1 deficiency (27,28). Our study was done in transgenic mice that express the human full-length AR with 97 glutamine residues (AR97Q) (11), the same mice previously used to test the effect of transgenic overexpression of mIGF-1 on SBMA pathogenesis (22). To provide clinically relevant results, we started treatment at age 10 wks, after the onset of disease manifestations (11). A randomized cohort of male SBMA mice received either vehicle or rhIGF-1/IGFBP3 (15 mg/kg) by daily intra-peritoneal injections for 6 wks. All investigators were blinded to the study agent versus vehicle until after the study was completed.

We first assessed whether treatment of SBMA mice with IGF-1 leads to activation of Akt in skeletal muscle. Akt activation was detected with a serine 473–phospho-specific antibody (29). By Western blotting analysis, we found that the IGF-1 treatment resulted in a significant increase in Akt phosphorylation at serine 473 (Figure 1A). These findings indicate that systemic treatment of SBMA mice results in activation of Akt in muscle.

Expansion of polyglutamine tracts leads to accumulation of mutant protein in aggregates and inclusions (30). We have previously shown that IGF-1 treatment of SBMA cells and transgenic overexpression of IGF-1 in skeletal muscle in SBMA mice decreases the aggregation of mutant AR (21,22). Therefore, we investigated whether systemic delivery of IGF-1 has an effect on mutant AR aggregation in the SBMA mice. Here, we define aggregates as high–molecular weight oligomers soluble in radioimmunoprecipitation assay buffer after high-speed centrifugation and sonication, which can

Figure 2. IGF-1 attenuates weight loss and enhances motor behavior of SBMA mice. (A) Two mice from the same litter at wk 16 of age, showing the gross appearance of an IGF-1–treated (left) and vehicle-treated (right) SBMA mouse. (B, C) Body weight and hanging wire performance of SBMA mice treated with IGF-1 (\( n = 20 \)) or vehicle (\( n = 20 \)). Scale bars, SEM. (D) TreadScan assessment showed improved motor performance in AR97Q mice treated with IGF-1 (\( n = 6 \)) compared with vehicle (\( n = 6 \)). Scale bars, SEM. *\( p < 0.05 \); **\( p < 0.01 \). (E) Kaplan-Meier survival curves of mice treated with IGF-1 (\( n = 20 \)) or vehicle (\( n = 20 \)). \( p = 0.09 \), log-rank test.
be detected as a smear in the stacking portion of SDS-polyacrylamide gels, as previously described (21,31,32). Western blot analysis showed that IGF-1 treatment reduces the accumulation of both monomeric and aggregated mutant AR in the skeletal muscle of AR97Q mice (Figure 1B). No changes were seen in the levels of endogenous mouse AR (Supplementary Figure S1). To assess whether IGF-1 affects the accumulation of mutant AR into nuclear inclusions in muscle, cross-sections of quadriceps muscle were stained with the polyglutamine-specific antibody 1C2, as previously described (22). The IGF-1 treatment reduced the number of 1C2-positive nuclei by about half (Figure 1C). Together, these results indicate that systemic treatment of SBMA mice with IGF-1 leads to Akt activation and reduction of aggregated mutant AR in muscle.

**IGF-1 Attenuates Disease Manifestations in SBMA Mice**

We next examined whether IGF-1 treatment ameliorates the disease phenotype in SBMA mice. Male transgenic and wild-type mice were randomly assigned to receive either vehicle or IGF-1 as described above from 10 through 20 wks of age. To assess the effect of IGF-1 on the disease manifestations, we analyzed weekly body weight (Figures 2A, B), motor function (Figures 2C, D) and survival (Figure 2E). IGF-1–treated SBMA mice had less body weight loss than vehicle-treated mice (p = 0.001; Figures 2A, B). Grip strength, assessed by a hanging wire test, significantly improved in the IGF-1–treated mice (p = 0.01; Figure 2C). Motor performance was analyzed using a TreadScan device at wks 10, 13 and 16. This gait analysis showed longer stride time (p = 0.01) and length (p = 0.01) and reduced rear track width (p = 0.009) and paw area (p = 0.04) in the IGF-1–treated SBMA mice compared with vehicle-treated mice at wk 16 (Figure 2D). To determine the effect of IGF-1 treatment on survival in this cohort of mice, lifespan was assessed up to a maximum of 45 wks. Mice treated with IGF-1 lived ~3 wks longer on average than vehicle-treated mice, although this did not reach statistical significance (p = 0.09; Figure 2E), perhaps because a subset of the mice were already quite weak and close to death at 10 wks when the treatment was initiated. A post hoc analysis excluding these mice (hunched posture and sustained weight loss >5% at wk 10) showed a significant difference between the remaining animals in the two groups (p = 0.02; Supplementary Figure S2). Taken together, our results demonstrate that rhIGF-1/IGFBP3 administration...
ameliorates disease manifestations and extends survival in early disease stage–treated AR97Q mice.

IGF-1 Reduces Muscle Pathology in AR97Q Mice

To investigate the effects of IGF-1 treatment on SBMA muscle pathology, quadriceps muscles from 16-wk-old SBMA mice and wild-type littermates treated with vehicle or IGF-1 were collected for histopathological analyses. Muscle cross-sections of vehicle-treated AR97Q mice stained with hematoxylin and eosin (Figure 3A) and nicotinamide adenine dinucleotide (Figure 3B) showed angulated myofibers and grouped atrophic fibers as well as enlarged fibers with central nuclei. These changes are indicative of both neuropathic and myopathic changes, as previously reported (22). These signs of muscle atrophy and degeneration were markedly reduced in the hrIGF-1/IGFBP3-treated mice, indicating a protective effect of IGF-1 on muscle pathology. To further investigate the effect of IGF-1 administration on AR97Q muscle, we analyzed muscle fiber size. In the IGF-1–treated SBMA mice, the mean cross-sectional area and the minimal Feret diameter of myofibers were significantly increased compared with vehicle-treated mice (p = 0.01 and p = 0.03, respectively; Figures 3C–E). Moreover, we performed gene expression analysis of transcripts associated with muscle denervation, including myogenin and Chrna1, which we previously showed to be upregulated in both SBMA mice and patients (22). IGF-1 treatment of SBMA mice resulted in reduced expression of myogenin and Chrna1 (p = 0.003 and p = 0.0009, respectively; Supplementary Figure S3). Collectively, these results show that systemic treatment of SBMA mice with IGF-1 attenuates muscle denervation and degeneration.

Systemic IGF-1 Treatment Has Indirect Effects on Motor Neurons in SBMA Mice

To characterize the effects of IGF-1 on the motor neurons of SBMA mice, we analyzed the spinal cord expression levels of choline acetyltransferase (ChAT), a marker for functional cholinergic neurons. ChAT levels were increased in IGF-1–treated mice compared with vehicle-treated animals (p = 0.01; Figure 4A). Also the number of Nissl staining positive neurons with a diameter larger than 25 μm in the anterior horn of the spinal cord was slightly increased in mice treated with IGF-1, although this did not reach statistical significance (p = 0.09; Figure 4B).

No changes in the levels of Akt phosphorylation (Figure 4C) and of mono-
meric and aggregated mutant AR (p = 0.18 and p = 0.13, respectively; Figure 4D) were detected in the spinal cord. Altogether these results support the idea that an increase in Akt activation in muscle with IGF-1 treatment has an indirect effect on motor neurons in the spinal cord of SBMA mice.

DISCUSSION

Since the identification of the causative mutation, much has been learned about the molecular mechanism of SBMA through both in vivo and in vitro studies. The AR is a member of the nuclear hormone receptor superfamily (33) and resides in the cytoplasm when inactive. Upon androgen binding, the AR dissociates from heat shock proteins in the cytoplasm, translocates into the nucleus, binds DNA and activates and represses target genes. These steps are important in SBMA pathogenesis (34). In addition, the AR undergoes posttranslational modifications, which can have an important impact on the toxicity of the mutant protein. One such posttranslational modification relevant for SBMA pathogenesis is phosphorylation. We have previously shown that, in cell culture, IGF-1 induces phosphorylation of mutant AR via Akt, blocks ligand binding and reduces its toxicity (21). SBMA mice overexpressing the muscle isoform of IGF-1 have a milder phenotype (22).

IGF-1, also known as somatomedin C, is a 70–amino acid peptide homologous to proinsulin. It is released from liver after growth hormone stimulation. In addition, IGF-1 is synthesized in a number of target tissues, where it is thought to act locally in a paracrine fashion. IGF-1 forms complexes with at least six IGF-1–binding proteins, which increase its half-life, with IGFBP3 binding the largest fraction (~75%). IGF-1 has substantial effects on skeletal muscle, promoting and regulating muscle growth and differentiation (35,36), and on motor neurons, improving sprouting, axonal growth and cell survival (37). In vivo studies have shown that IGF-1 prevents motor neuron death after nerve injury in neonatal rats (38,39) and slows motor neuron disease progression in the wobbler mouse (40).

For its combined effects on neurons and muscle, human motor neuron diseases, such as amyotrophic lateral sclerosis, present an ideal opportunity for therapeutic intervention with IGF-1. However, to date, the response of peripheral delivery of IGF-1 has been not beneficial in amyotrophic lateral sclerosis clinical trials (41–43). SBMA may be a better candidate for IGF-1 administration, because IGF-1 specifically reduces the toxicity of mutant AR via Akt phosphorylation (21,22) and because muscle, which is readily accessible to systemic IGF-1 treatment, is likely to be involved in the pathogenesis of SBMA. Muscle biopsies from SBMA patients show myogenic changes as well as neurogenic atrophy (44), muscle pathology precedes spinal cord pathology in a knock-in mouse model of SBMA (45) and muscle-specific overexpression of wild-type AR leads to an SBMA-like phenotype (46). Therefore, it is appropriate to target muscle with peripheral delivery of IGF-1 in SBMA.

In this study, we evaluated the effects of daily intraperitoneal injection of IGF-1/IGFBP3 in a mouse model of SBMA. The combination of IGF-1 and IGFBP3 mimics the biological effects of IGF-1 alone but has a more favorable pharmacokinetic profile and less adverse effects (26).

The treatment improved motor performance, attenuated weight loss, improved muscle pathology and increased survival in AR97Q mice. It also had indirect neurotrophic effects on motor neurons. Skeletal muscle is well known to be a source of signals that are retrogradely transported by motor neurons (47) and influence motor neuronal survival, growth and maintenance (48). Our results indicate that systemic delivery of IGF-1 can modify AR polyglutamine toxicity in vivo. Because IGF-1 supplementation is not known to affect male fertility, testosterone levels were not measured in our cohort of mice.

Notably, in this study, we adopted an approach relevant to the clinical setting. Treatment allocation was randomized, and investigators were kept blind throughout the study. Also, the injections were started after the onset of disease manifestations. This is especially important for a disease such as SBMA, which is generally not diagnosed until after the onset of overt weakness.

CONCLUSION

Our study indicates that IGF-1 administration is beneficial in SBMA mice. These results provide a preclinical basis for further examining IGF-1 in patients with SBMA.

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DISCLOSURE

The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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