

IPLEX Administration Improves Motor Neuron Survival and Ameliorates Motor Functions in a Severe Mouse Model of Spinal Muscular Atrophy

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Spinal muscular atrophy (SMA) is an inherited neurodegenerative disorder and the first genetic cause of death in childhood. SMA is caused by low levels of survival motor neuron (SMN) protein that induce selective loss of α -motor neurons (MNs) in the spinal cord, resulting in progressive muscle atrophy and consequent respiratory failure. To date, no effective treatment is available to counteract the course of the disease. Among the different therapeutic strategies with potential clinical applications, the evaluation of trophic and/or protective agents able to antagonize MNs degeneration represents an attractive opportunity to develop valid therapies. Here we investigated the effects of IPLEX (recombinant human insulinlike growth factor 1 (rhIGF-1) complexed with recombinant human IGF-1 binding protein 3 (rhIGFBP-3)) on a severe mouse model of SMA. Interestingly, molecular and biochemical analyses of IGF-1 carried out in SMA mice before drug administration revealed marked reductions of IGF-1 circulating levels and hepatic mRNA expression. In this study, we found that perinatal administration of IPLEX, even if does not influence survival and body weight of mice, results in reduced degeneration of MNs, increased muscle fiber size and in amelioration of motor functions in SMA mice. Additionally, we show that phenotypic changes observed are not SMN-dependent, since no significant SMN modification was addressed in treated mice. Collectively, our data indicate IPLEX as a good therapeutic candidate to hinder the progression of the neurodegenerative process in SMA.

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INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive human disorder characterized by selective loss of α -MNs in the anterior horn of the spinal cord and by skeletal muscle atrophy. The degeneration of lower MNs causes progressive atrophy of the proximal muscles of

the limbs and trunk, leading to death by respiratory failure (1).

SMA is caused by homozygous loss or mutations of survival motor neuron 1 (*SMN1*) gene (2–3). The human genome contains two copies of the *SMN* gene: *SMN1* and *SMN2* (4). The *SMN2* gene is nearly identical to *SMN1*: the critical dif-

ference between these two genes consists in a C to T transition in exon 7 of *SMN2*. Consequently, exon 7 is alternatively spliced (that is, skipped) in most of the events, resulting in a functionally defective and highly unstable form of the protein (5). In addition, *SMN2* pre-mRNA is still able to undergo a correct splicing and to produce a small amount of full-length transcript and protein necessary to prevent embryonic lethality, but not sufficient to compensate for the loss of *SMN1* (6–7).

SMN is the core component of a large macromolecular complex, called SMN complex, composed of eight additional proteins: Gemin 2–8 and unrip (8). The complex mediates the biogenesis of small nuclear ribonucleoproteins (snRNPs), the building blocks of spliceosome. In the cytoplasm, the SMN complex chaperones

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the efficient assembly of Sm proteins around a conserved sequence (Sm site) of small nuclear RNAs (snRNAs), such as U1, U2, U4, U5, U11, U12 and U4atac, generating snRNPs (9–10). SMN has been proposed to function in several RNA metabolic pathways, including the assembly of snRNPs of the LSm class (11) and messenger ribonucleoproteins (mRNPs), related to LSm (12). Additionally, SMN have been proposed to function in an axon-specific complex, including hnRNPQ/R and ZBPs proteins, suggested to work in axonal mRNA traffic (13–14). Although SMN is an ubiquitously expressed protein, why the disease selectively affects MNs is still unclear.

To date, there are no treatments to slowdown or to reverse neurodegeneration in SMA, therefore the identification of therapeutics or new intervention strategies is strongly needed. Currently, among the different methodologies under investigation (15), the identification and effectiveness evaluation of neurotrophic and neuroprotective agents antagonizing MNs loss represents a valid approach (16–20).

Insulinlike growth factor-1 (IGF-1) is a multifunctional factor member of the insulin family. The circulating IGF-1 isoform, mainly synthesized in the liver, is a ternary complex composed by IGFbps and the acid-labile subunit (Igfals) (21). IGF-1 is active in several physiological processes, among them, it plays an important role in the development and survival of spinal MNs (22–23) as well as in maintaining muscle integrity and regeneration after injury and denervation (24). For instance, muscle-restricted expression of IGF-1 was shown to support muscle integrity, stabilize neuromuscular junctions and enhance motor neuron survival in SOD1/G93 mouse model of amyotrophic lateral sclerosis (ALS), delaying the progression of the disease (25). *In vitro* and *in vivo* experiments demonstrated the ability of IGF-1 to enhance spinal motor neuron survival and axon outgrowth during corticospinal motor neuron development (23). *In vivo* IGF-1

overexpression, by viral vector approaches, was able to mitigate ALS pathological phenotype and also to lessen phenotypic manifestation in a mouse model of spinal and bulbar muscular atrophy (26). Furthermore, the specific overexpression of IGF-1 in the skeletal muscle of SMA mice mitigates SMA phenotype, even if the motor behavior does not seem to be improved significantly (27).

IPLEX is free recombinant human IGF-1 (rhIGF-1) complexed with IGF-binding protein 3 (rhIGFBP-3), one of six naturally occurring binding proteins that have been shown to cross the blood brain barrier (28). The addition of the natural binding protein, BP-3, allows a significant increase of half-life and consequently higher dosing of circulating IGF-1. Administration of IGF-1 in this form not only may enhance the bioavailability of IGF-1, but also may prevent hypoglycemia when given at higher doses, because the IGF-1/BP3 complex does not interact readily with insulin receptors (29). IPLEX is available commercially and employed to treat short growth failure in children and adolescents with severe primary deficiency (30). When used in an open-label trial for myotonic dystrophy type 1 patients, it was generally well tolerated and patients showed an increased lean body mass and improvement, but no increase in muscle strength or function (31).

In this study, we investigated potential therapeutic effects of IPLEX (that is, IGF-1) in a severe mouse model of SMA. In particular, we administered mice starting at postnatal d 2 to d 10 (P2–P10) and we then analyzed several molecular and behavioral parameters within the same time window.

MATERIALS AND METHODS

Animal Models

Breeder pairs for SMN Δ 7 SMA mice on a FVB background (stock number 005025; FVB.Cg-Tg(SMN2*delta7)4299Ahmb Tg(SMN2)89Ahmb Smn1tm1Msd/J) were purchased from Jackson Laboratories

(Bar Harbor, ME, USA). Tail-purified DNAs were used for mouse genotyping as described in Le *et al.* (32). All *in vivo* studies were carried out in accordance with European Economic Community Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987 (33) and with the NIH-used *Guide for the Care and Use of Laboratory Animals* (34).

Drug Formulation

IPLEX (mecasermin rinfabate [rDNA origin] injection) is a product of Inmed Incorporated (Richmond, VA, USA). It is a binary protein complex of human insulinlike growth factor-1 (rhIGF-1) and human insulinlike growth factor-binding protein-3 (rhIGFBP-3) in aqueous solution (60 mg/mL).

Determination of IGF-1 Circulating Levels

Animals were euthanized and blood collected with a Hamilton syringe, as soon as possible from P10 mouse hearts. Blood samples were incubated 1 h at room temperature to allow the formation of blood clot. Following centrifugation (15 min, 2,000g), mouse sera were recovered and stored at -80°C .

Mouse IGF-1 levels were assessed from animal sera (WT and SMA, $n = 10$) with Mouse/Rat IGF-1 Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., Minneapolis, MN, USA). rhIGF-1 and rhIGFBP-3 were analyzed by solid-phase enzyme-labeled chemiluminescent immunometric assay (IMMULITE 2000 IGFBP-3 and IMMULITE 2000 IGF-1, Diagnostic Products Corporation, Los Angeles, CA, USA) from mouse sera (SMA IPLEX $n = 10$; WT negative control $n = 5$).

Neonatal Drug Administration

SMA and carrier littermates were assigned randomly to receive IPLEX (60 mg/kg/day) or vehicle via intraperitoneal injection from P2 to P10. The SMA and WT vehicle groups received equal volumes of saline solution. Total injected volumes were 30 μL . The date of birth was designated as P1.

Behavior

Body weight of animals was measured daily starting from the date of birth (P1) (WT: $n = 6$; SMA vehicle: $n = 36$; SMA IPLEX: $n = 50$). Righting reflex (35–36) was evaluated at P7, P8 and P9 (WT: $n = 6$; SMA vehicle; $n = 33$; SMA IPLEX: $n = 46$). Negative geotaxis responses were measured at P9 (WT: $n = 6$; SMA vehicle: $n = 27$; SMA IPLEX: $n = 26$). Mice performance was scored according to their ability to turn around and climb an inclined grid (40°) within 60 s. Score 0: mice not even able to stand on the grid. Score 1: mice failed to accomplish the test. Score 2: mice able to turn 90°. Score 3: mice able to turn 180°. Score 4: normal geotaxis response (33–34). Functional assessments were carried out in a double-blind experimental design.

Gene Expression Analyses

Total RNAs from spinal cords, muscles and livers were extracted with TRIzol Reagent (Invitrogen; Life Technologies Corporation, Carlsbad, CA, USA) following manufacturer's instructions. DNase I (RNase-free) (Ambion; Life Technologies Corporation) was then applied to eliminate genomic DNA contamination from total RNA samples.

One μg of RNA was reverse transcribed with the High-Capacity cDNA Archive kit (Life Technologies Corporation) and used in real-time reverse transcription (RT)–polymerase chain reaction (PCR). mRNAs were measured by SYBR Green or TaqMan chemistry (Life Technologies Corporation) using primers and probes reported in Table S1. The comparative $\Delta\Delta\text{Ct}$ method was used to quantify relative gene expression levels.

Western Blot Analyses

Spinal cords and skeletal muscles (quadriceps) of P10 mice were homogenized in RIPA buffer (50 mmol/L Tris:HCl pH 7.4; 150 mmol/L NaCl; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS) containing complete Mini EDTA-free Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland).

Total protein extracts were resolved on 12% SDS-PAGE gels and analyzed by Western blot. Nitrocellulose membranes were saturated in 5% milk/PBS and then probed with mouse anti-SMN antibody (1:10000; BD Transduction Laboratories; BD, Franklin Lakes, NJ, USA) and mouse anti- α -tubulin antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Peroxidase-conjugated secondary antibodies were used (1:10000; EMD Millipore Corporation, Billerica, MA, US), and the signal was detected with an ECL detection kit (Amersham; GE Healthcare, Fairfield, CT, USA). Densitometric analyses were carried out using ImageJ software (NIH, Bethesda, MD, USA; <http://rsbweb.nih.gov/ij/>) according to standard procedures.

snRNP Level Analysis

snRNAs were quantified by SYBR Green chemistry real time RT-PCR (Power SYBR Green PCR Master Mix, Life Technologies Corporation). Total RNA was purified as described previously. snRNA specific reverse primers and 5.8S rRNA (used as reference) were used to generate cDNA using High-Capacity cDNA Archive kit (Life Technologies Corporation). The same reverse primers were used in RT and also in real-time PCR.

Histological and Immunofluorescence Analysis

Segments of tibial muscles from WT, SMA vehicle and SMA IPLEX mice were embedded in tissue freezing medium and snap frozen in nitrogen-cooled isopentane.

Frozen muscle sections (7 μm) were used for immunofluorescence analysis. Briefly, sections were fixed in 4% paraformaldehyde for 10 min on ice washed in PBS with 1% BSA and 0.2% Triton X-100, preincubated for 1 h in 10% goat serum at RT, and incubated overnight at 4°C with polyclonal antibody against Laminin (Sigma-Aldrich, St. Louis, MO, USA).

For labeling MNs in spinal cord slices, spinal cords from P10 mice were removed surgically, fixed in 4% paraformal-

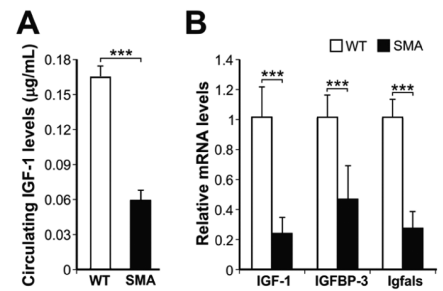


Figure 1. IGF-1 is strongly impaired in SMN Δ 7 SMA mice. (A) IGF-1 circulating levels were analyzed by ELISA in P10 WT and SMA mouse sera and reported as absolute quantification. (B) RT-qPCR analyses of IGF-1, IGFBP-3 and Igfals mRNAs levels in the liver of P10 WT and SMA mice. Data are reported as mean \pm SEM of 10 animals of each group. (*** $P < 0.001$)

dehyde, washed in PBS and processed as reported above. Spinal cord slices ($n = 6$ mice/group) were then incubated overnight at 4°C with SMI-32 monoclonal antibody (1:1000; DAKO, Glostrup, Denmark) and nuclei counterstained with Hoechst. Inverted microscope (Axioskop 2 plus; Carl Zeiss AG, Oberkochen, Germany) using 20 or 40 \times lenses, was used. Images were processed using Axiovision 3.1 (25,37).

Morphometric Analysis

Cross-sectioned tibialis anterior (TA) muscles of each mouse group (WT, SMA vehicle and SMA IPLEX; five mice/group) were photomicrographed and muscle section fibers analyzed using Scion Image Software (v. b 4.0.2; Scion Corporation, Frederick, MD, USA).

Statistics

Statistical analysis was performed with GraphPad Prism v 5.0 software; groups were compared by Mann-Whitney rank sum test and the difference in the median values between the three groups was considered significant for $P < 0.05$.

RT-qPCR statistical significance was determined using the Student t test.

Two-way analysis of variance (ANOVA) for repeated measure was

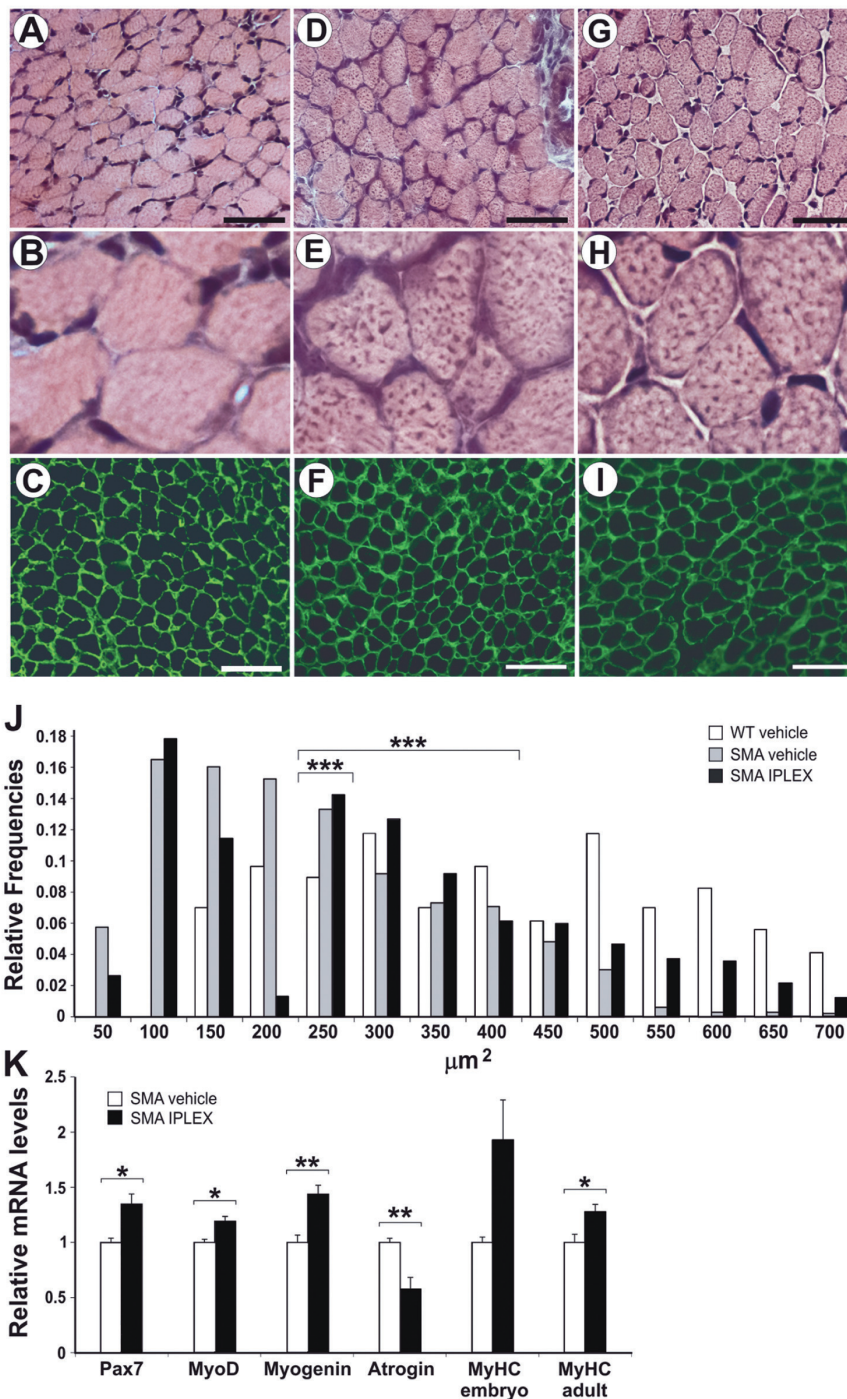


Figure 2. IPLEX treatment attenuates muscle wasting in a mouse model of SMA. Hematoxylin and eosin-stained cross-section histological analysis of TA muscles at P10 in WT (A), SMA vehicle (D) and SMA IPLEX (G). Panels (B), (E), (H) show magnifications of WT, SMA vehicle and SMA IPLEX respectively. Immunofluorescence staining of Laminin in TA cross-section of WT (C), SMA vehicle (F) and SMA IPLEX (I), scale bar = 100 μm . (J) Frequency distribution of fiber size in TA muscles of WT, SMA vehicle and SMA IPLEX mice, Mann-Whitney rank sum test: $***P < 0.001$. (K) mRNA levels were analyzed by real time RT-PCR in quadriceps isolated from mice daily treated with vehicle or IPLEX (60 mg/kg) on P2-P10. Data are representative of 10 mice. Values represent mean \pm SEM. $*P < 0.05$; $**P < 0.01$.

used to analyze mean body weight and righting reflex in the three groups (WT, SMA vehicle and SMA IPLEX) across 3 d (d 7, d 8 and d 9).

One-way ANOVA was used to compare negative geotaxis score between groups. *Post hoc* was performed using Bonferroni test. P was set < 0.05 .

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

RESULTS

IGF-1 Circulating Levels Are Strongly Impaired in SMA Mice

Before IPLEX administration, we considered possible alterations of endogenous IGF-1 levels that could void the relevance of the drug test, in particular we were interested in excluding potential epiphenomenal IGF-1 upregulation.

So, we first measured the IGF-1 circulating levels comparing WT mice to SMA littermates at P10; mice were bled and blood samples processed to isolate sera that were then analyzed by ELISA. Unexpectedly, this analysis revealed that SMN Δ 7 SMA mice show markedly reduced amount of circulating IGF-1 levels compared with WT mice (Figure 1A). Thus the impairment of this trophic factor increased the relevance of testing IPLEX in SMA mice.

Circulating IGF-1 is mainly produced in liver together with IGFBP-3 and Igfals carrier proteins, that function to increase IGF-1 stability. We reasoned that IGF-1 protein reduction could result from decreased mRNA levels as well as from changes in the expression of carrier proteins resulting in reduced stability of that factor. For this reason we measured mRNA levels of IGF-1, IGFBP-3 and Igfals in the liver of P10 WT and SMA mice. RT-qPCR analyses reported in Figure 1B indicate that, in the liver, the expression of the three genes encoding the IGF-1 circulating complex is reduced strongly, suggesting the impairment of transcriptional activity or posttranscriptional events of part of the IGF-1 pathway in P10 SMA mice. Very recently,

during the preparation of this manuscript, Hua *et al.* (38) reported analogous defects in a different mouse model of SMA, confirming our findings.

Since IPLEX was administered by intraperitoneal injection we evaluated the proper drug delivery to animal blood. To do so, we assessed the rhIGF-1 and rhIGFBP-3 levels from untreated and treated P10 mice 2 h after injection, confirming that IPLEX reach mouse circulation ($4 < \text{rhIGF-1} < 7 \mu\text{g/mL}$; $20 < \text{rhIGFBP3} < 40 \mu\text{g/mL}$ in serum) without critical differences between WT and SMA mice. No cross-reaction of murine protein was noticed, indicating that the measured IGF-1 levels only depend on IPLEX. Importantly, we evaluated potential toxic effects of rhIGF-1 high levels by histological analyses. In particular, we carried out macroscopic and microscopic examination of different organs (liver, spleen and kidney) comparing tissues isolated from SMA mice that received IPLEX or vehicle. We noticed an absence of macroscopic alterations, including changes of size and weight of organs. Additionally, microscopic examinations revealed absence of pathologic changes in terms of inflammatory infiltrates, signs of cell damage, swelling, necrosis or cell shape alterations.

SMNΔ7 SMA Mice Are Responsive to IPLEX Administration That Results in Amelioration of Muscular Phenotype

To assess the responsiveness of SMA mice to human IGF-1, we assayed changes in muscles as one of the most studied IGF-1 target tissue. Following intraperitoneal injection of IPLEX (60 mg/kg) in mice from P2 to P10, we carried out histological (Figures 2A–B, D–E, G–H) and morphometric (Figures 2C, F, I) analyses on TA muscles. SMNΔ7 SMA mice (Figures 2D, J) showed significant reduction in cross-sectional area (CSA) of TA skeletal muscles fibers compared with their age-matched WT sibs (Figures 2A, J). The frequency distribution of CSA (Figure 2J) reveals a shift of the median values toward the small size of muscle fibers in all the animals analyzed (n = 5). In contrast, IPLEX adminis-

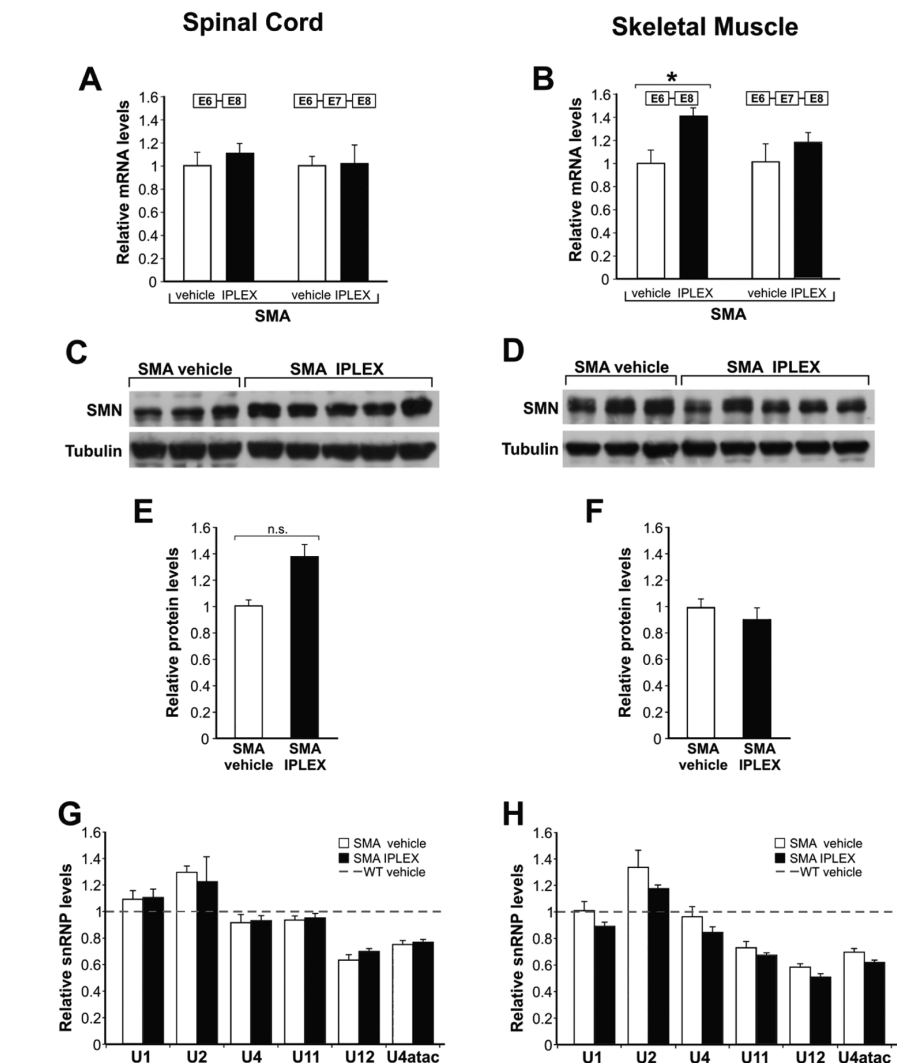


Figure 3. IPLEX does not affect the transcription, protein levels and activity of SMN in spinal cord and skeletal muscles of SMA mice. (A) and (B): Real time RT-PCR analyses of SMNΔ7 and full length mRNAs were carried out on mouse tissues. Values represent mean ± SEM. *P < 0.05. (C) and (D): Western blot analysis of equal amount of proteins from SMA mice, injected with vehicle or IPLEX. (E) and (F): Relative quantification of SMN protein levels by densitometric analysis. Total RNAs from the spinal cord (G) and quadriceps (H) of P10 WT and SMA mice (treated with vehicle or with IPLEX) were analyzed by real-time RT-PCR for quantifying the levels of specific snRNAs. Dashed gray line corresponds to snRNA levels of WT mice treated with vehicle, used as references. Data are representative of three independent biological replicates. Values represent mean ± SEM. *P < 0.05; n.s. = not significant.

tration (Figures 2G, J) induced a significant increase of the median values of single fibers cross-sectional area (WT: $407.08 \mu\text{m}^2 \pm 14.81$; SMA: $212.0 \mu\text{m}^2 \pm 2.58$; SMA IPLEX: $281.07 \mu\text{m}^2 \pm 5.05$) ($P < 0.001$). Figures 2B, E and H represent magnifications of WT, SMA treated with vehicle and with IPLEX, respectively.

We then isolated quadriceps from P10 SMA mice treated with IPLEX or vehicle and the expression of muscle specific markers were measured by RT-qPCR (Figure 2K). mRNA expression levels of Pax7, MyoD and Myogenin were found significantly increased in SMA mice that received IPLEX compared with the con-

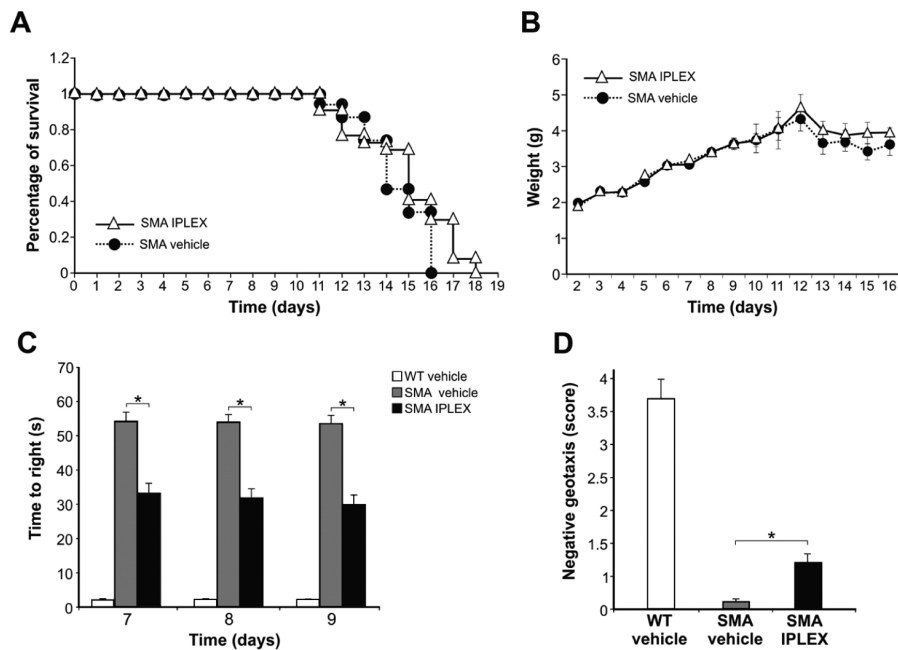


Figure 4. Administration of IPLEX does not increase the survival and body weight of SMA mice, but it improves the motor behavior of SMA mice. (A) Kaplan-Meier survival curves of SMA mice treated with IPLEX ($n = 22$) or vehicle ($n = 15$). (B) Body weight time course analysis of SMA mice treated with vehicle and SMA mice treated with IPLEX. (C) Mice were assessed from d 7 to d 9 for their ability and inability to right themselves when placed on their backs on a level surface (Righting Reflex). Values represent mean \pm SEM, comparing SMA IPLEX to vehicle treated mice; $*P < 0.05$. (D) P9 WT and SMA mice treated with vehicle or IPLEX were tested for their latency to complete the geotaxis test (Negative Geotaxis). Values represent mean \pm SEM, comparing SMA IPLEX to SMA vehicle treated mice, $*P < 0.05$. SMA IPLEX versus SMA vehicle, Bonferroni *post hoc* test.

control group, suggesting potential changes in myosatellite cells. Additionally, we found that IPLEX administration induces a significant increment of the MyHC adult isoform (the major component of contractile apparatus) that was reported to be deficient in SMA mice (39). We also observed a marked variability in the expression of MyHC embryonic isoform (Figure 2K), however statistical analysis of RT-qPCR data does not support a consistent expression pattern. Furthermore, we evaluated potential changes of atrogenin-1, a key factor involved in skeletal muscle atrophy, which is negatively modulated by IGF-1 (40). Atrogenin-1 expression coincides with loss of muscular mass and represents a relevant component of the enhanced proteolysis in atrophy (41). Importantly, mice treated with IPLEX showed an evident

reduction of atrogenin-1 expression, suggesting changes in muscular atrophy progression (Figure 2K).

Collectively, these data indicate that SMA mice are responsive to human IGF-1 (that is, IPLEX) that is able to induce reduction of muscle waste suggesting a slowdown of atrophy progression.

IPLEX Administration Does Not Affect SMN Biochemical Activity

To evaluate whether IPLEX administration influences SMN expression, we measured SMN full length and $\Delta 7$ mRNA levels in spinal cord (Figure 3A) and muscle (Figure 3B) of treated SMA mice at P10. RT-qPCR analyses do not show any increase of full length SMN mRNA in either muscle or in spinal cord. Curiously, IPLEX administration produces in muscle a $\sim 40\%$ ($P < 0.05$) in-

crease in SMN $\Delta 7$ mRNA expression (Figure 3B), probably as a consequence of enhanced transcription of one or more SMN $\Delta 7$ transgene copies. Additionally, we evaluated a possible accumulation of SMN protein, so spinal cord (Figure 3C) and skeletal muscle (Figure 3D) were investigated by Western blot and further analyzed by densitometry (Figures 3E and F, respectively) comparing treated SMA mouse tissue extracts with untreated ones. Figure 3E shows a slight accumulation of SMN protein only in the spinal cord of IPLEX treated mice, that is, however, not statistically significant.

To address whether or not IPLEX could enhance SMN complex biochemical activity and possibly induce changes in mouse phenotypes, we assessed the snRNP relative levels in spinal cord (Figure 3G) and skeletal muscle (Figure 3H) of IPLEX or vehicle-treated animals using WT mice as reference. To date, the SMN function in snRNP assembly represents the only SMA molecular defect that can be quantified (42,43). To do so, we measured by RT-qPCR the steady state levels of several U snRNAs reported to be affected in SMA animal models (42,43). The analysis confirms the alteration of snRNP profile in SMA animals and reveals no changes in SMA-treated mice (Figures 3G, H). Thus, in this context, we cannot attribute a role to SMN in eventual SMA mouse phenotypic changes.

IPLEX Administration Does Not Improve Mouse Survival but Does Ameliorate Neuromuscular Functions in SMN $\Delta 7$ SMA Mice

Mouse survival, body weight and motor functions were tested in time course analyses on IPLEX-treated mice. Figure 4A reports the Kaplan-Meier survival curve of mice treated with IPLEX compared with the SMA control group, no significant survival improvement was noticed following daily drug administration. Additionally, no body weight increase was evident for SMA IPLEX mice whose trend overlaps the reference group (Figure 4B). One-way ANOVA for

repeated measures reveals a significant effect of groups ($F_{2,86} = 6.93$; $P = 0.001$), and days ($F_{2,172} = 13.426$; $P < 0.0001$), but not any significant interaction effect.

Conversely, IPLEX-treated mice show a significant motor function improvement when compared with SMA vehicle-treated animals. In particular, mice were assayed for righting reflex at P7–P9 (Figure 4C). One-way ANOVA for repeated measures reveals a significant effect of group $F_{2,83} = 43.08$; $P < 0.0001$). Bonferroni *post hoc* analysis clearly shows that the SMA IPLEX group performed significantly better than the SMA vehicle group on each testing day (d 7 $P = 0.003$, d 8 $P = 0.0003$ and d 9 $P = 0.0009$, SMA IPLEX versus SMA vehicle). Furthermore, negative geotaxis response at P9 in SMA mice that received IPLEX (Figure 4D) was measured and found significantly enhanced when compared with the SMA control group ($P = 0.003$), confirming a positive trend of neuromuscular performances. Together, these results indicate that daily administration of IPLEX immediately after birth ameliorates neuromuscular functions in SMA mice.

IPLEX Administration Slows Down Motor Neuron Degeneration in SMNΔ7 SMA Mice

We next investigated the potential ability of IPLEX to protect MNs from degeneration in a SMA environment, thus we studied spinal cords of P10 WT, SMA vehicle and SMA IPLEX-treated mice. We analyzed the ventral horns of 10 spinal cord sections (thickness greater than 25 μm) per mouse (n = 6); additionally, nonconsecutive sections were considered to cover almost the whole lumbar spinal cord. To do so, we employed SMI-32 antibody to stain nonphosphorylated neurofilament (NF-H) able to evidence large MNs in spinal cord slices. NF-H colocalizes with other well-established motor neuron markers (including acetylcholine, calcitonin gene-related peptide and peripherin) (37). MNs were identified considering cell shape and size. In particular, we included in the analysis SMI32-positive

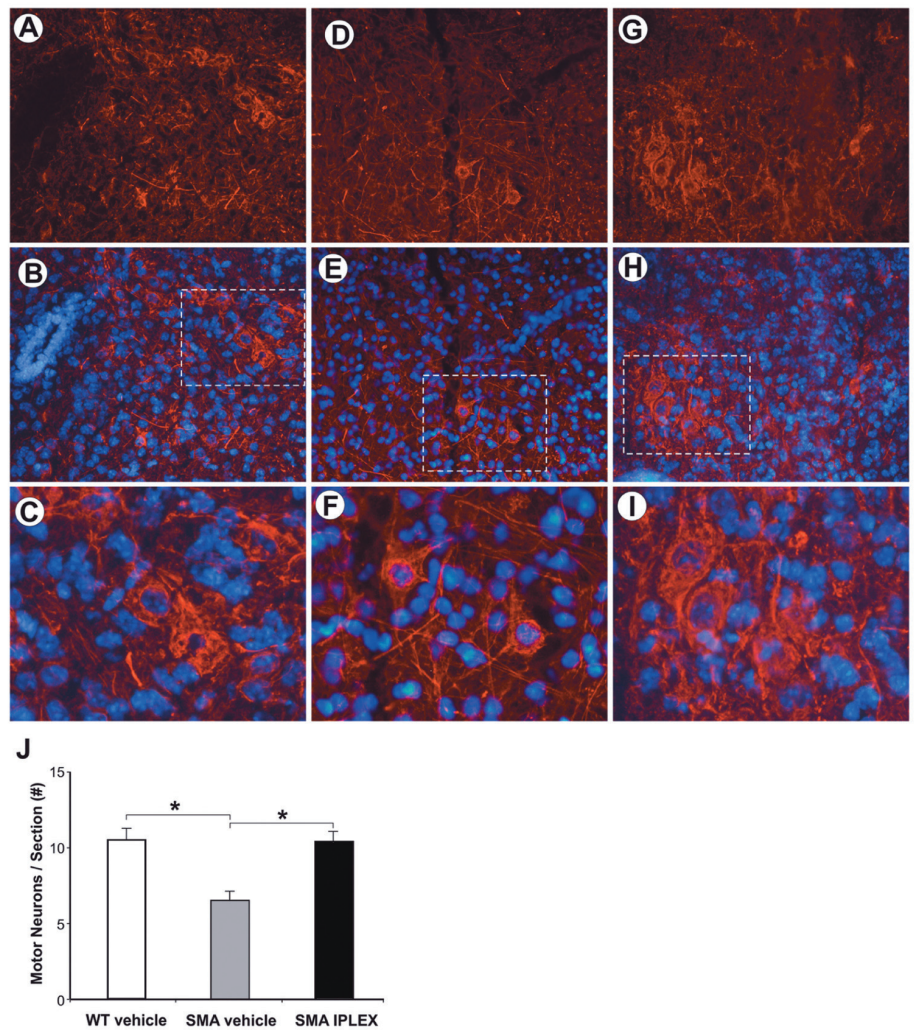


Figure 5. IPLEX prevents loss of MNs in the spinal cord of SMA mice. NF-H immunostaining in spinal cord ventral horn of WT (A, B, C), SMA vehicle (D, E, F) and SMA IPLEX (G, H, I) P10 mice. Figures 5B, E and H show Hoechst counterstaining. Figures 5C, F and I represent a particular of Figures 5B, E and H, respectively. (J) MNs mean number for section of WT, SMA vehicle and SMA IPLEX mice. Data were reported as mean ± SEM, Mann-Whitney rank sum test: * $P < 0.02$.

cells with diameter greater than 25 μm (44) that specifically localize in the ventral horns of spinal cord sections. Additionally, we excluded from MN count those cell bodies without an evident Hoechst nuclear counterstaining. Immunohistochemical analyses of ventral spinal cords (Figure 5) revealed that SMNΔ7 SMA mice (Figures 5D, E, F) show a reduced number of MNs at P10 (Figure 5J). Specifically, we observed a 38.09% decrease in the mean number of MNs when SMA mice were compared with WT littermates

(Figures 5A, B, C, J) (n = 6; WT: 10.5 ± 0.76; SMA vehicle: 6.5 ± 0.67). Strikingly, IPLEX treatment prevents motor neuron loss in 10 d old SMA mice (Figures 5G, H, I, J). The average number of MNs per section in the spinal cord of SMNΔ7 SMA mice administered with IPLEX does not show significant differences from WT littermates (Figure 5J; n = 6; WT: 10.5 ± 0.76; SMA IPLEX: 10.33 ± 0.8).

These data suggest that IPLEX could represent a factor contributing to support motor neuron survival.

DISCUSSION

IGF-1 is a critical player in several physiological processes, especially in myogenesis and CNS development (23) and it also is implicated in the control of skeletal muscle and brain homeostasis (45). IGF-1 plays an important role in the nervous system, stimulating postnatal brain growth (46), promoting neuron survival and growth (47,48), increasing the rate of axon regeneration in crush-injured sciatic nerve (49) and increasing the proliferation of oligodendrocytes (50). Lack of IGF-1 in knockout mice severely affects survival and maturation of neurons (51).

In this work, we investigated the therapeutic potential of IPLEX, a protein complex consisting of recombinant hIGF-1 and one of its binding protein hIGFBP-3, on a severe mouse model of SMA. Notably, rhIGF-I complexed to rhIGFBP-3, was developed in an attempt to prolong the half-life of IGF-1 and potentially reduce side effects (52).

During our study we found an unexpected deficiency of circulating IGF-1 in SMA mice. This intriguing observation conferred additional importance to IPLEX administration. Further analyses, carried out into the liver as the first tissue responsible for circulating IGF-1 synthesis, elicited that IGF-1 mRNA levels are strongly impaired in SMA mice (almost five-fold lower). Recently, during the preparation of this manuscript, Hua *et al.* also have reported pronounced reduction of circulating IGF-1 levels in a different severe SMA mouse model, confirming our data. Interestingly, in this paper, the authors show that restoration of SMN levels, through induced inclusion of *SMN2* exon 7, is able to recover IGF-1 circulating levels, suggesting a direct or indirect correlation between SMN and IGF-1 levels. Importantly, the rescue of IGF-1 levels, following SMN restoration, strongly supports IGF-1 as potential biomarker with important applications in clinical trials, that, however, requires validations in human patients. Moreover, Hua *et al.* (38) assessed the levels of IGF-1, IGFBP-3 and Igfals of P1 and P5 mice,

finding that only Igfals is impaired. These results suggested a critical role that Igfals plays stabilizing IGF-1. In our study, we found that in the liver of P10 SMA mice not only IGF-1 mRNA levels are impaired but also IGFBP-3 and Igfals are reduced strongly, possibly contributing to exacerbate the IGF-1 defect.

We then evaluated the effects of daily intraperitoneal administration (between P2 and P10) of IPLEX in SMA mice, studying potential phenotypic changes within the injection time window. In particular, IPLEX-treated mice showed a statistically significant increase of the average size of muscular fibers, indicating that SMA mice are responsive to human IGF-1. Additionally, we observed a reduction of atrogen-1 expression levels, possibly implicating an attenuation of muscular atrophy in SMA-treated mice. Atrogen-1 appears to be a critical factor leading to muscle atrophy in diverse diseases; indeed, atrogen-1-deficient mice are resistant to muscle atrophy (53). Moreover, when compared with the SMA control group, mice administered with IPLEX showed the upregulation of MyHC expression, suggesting an improvement in maturity of muscle fibers (39).

We also considered the possibility that IPLEX, directly or indirectly, may affect SMN mRNA expression, protein levels or SMN complex biochemical activity, so we analyzed tissues of control and treated SMA mice (that is, skeletal muscle and spinal cord). Even if no significant increase in mRNAs was observed, except for the $\Delta 7$ isoform in muscle, we detected a slight and not statistically significant increase of SMN protein in spinal cord of IPLEX-treated animals. However, the degree of protein accumulation necessary to efficiently recover or improve SMA phenotypes is still not clear; actually, it is well established that a heterozygous subject is asymptomatic, indicating that, theoretically, 50% protein is sufficient to prevent the disease and possibly sufficient for a full rescue. SMN protein functions in the biogenesis of snRNPs. Recent works demonstrated the accumulation of defects in snRNP levels

in tissues of SMA mice (42,43), defects that we confirmed in our hands and that we employed as readout to assess the SMN complex activity in IPLEX-treated animals. In particular, we asked whether or not IPLEX could somehow stimulate the complex activity. No snRNP correction was detected, suggesting that phenotypic changes could not be attributed directly to SMN changes.

Although IPLEX administration, limited to the first 10 d of animal life, does not improve the survival of animals significantly and no gain of body weight was observed, two different behavioral tests, representative of amelioration in motor function (that is, righting reflex and negative geotaxis) were carried out during the same period. Importantly, in both tests, performances of mice that received IPLEX were found to be improved significantly, indicating that drug administration triggers a motor behavioral amelioration in severe mouse model of SMA.

Recently Bosch-Marce *et al.* reported the effects of IGF-1 muscle-restricted overexpression in *SMN Δ 7* SMA mouse model, actually the same model used in this work. In that study, SMA mice were crossed with a strain where IGF-1 expression is under the control of myosin light chain promoter, resulting in an animal model that showed increased muscle mass and a moderate extent in lifespan, but no detectable improvement in motor function (27).

Additionally, Shababi *et al.* (54) showed that delivery of IGF-1 cDNA, through intracerebroventricular injection, is able to induce amelioration of SMA phenotype in terms of lifespan and gain of body weight in a more severe SMA mouse model. However, despite the approaches employed (that is, transgenesis, plasmid DNA delivery and systemic protein administration), IGF-1 seems to induce positive changes in the SMA environment.

In SMA, motor neuron dysfunction and loss represent critical events during pathology. Here we found that IPLEX is able to prevent motor neuron loss; in fact

no significant differences were measured between WT and SMA-treated animals, when analyzed for NF-H expression (37).

CONCLUSION

Nevertheless, according to our results, it is evident that motor neuron preservation *per se* does not rescue the course of the pathology and that SMA is caused primarily by impaired MNS, so their degeneration could be a later event exacerbating the end stage phenotypes. Recent studies, carried out on the same mouse model employed in our work, revealed the accumulation of defects localized in neuromuscular junctions (NMJs), such as poor terminal arborization and neurofilament accumulation at distal end of α -motor axons (38,55–56). Temporally, these events were observed to precede loss of somata before the disease phenotype become apparent. However, according to our data, the persistence of normal neuron number, together with the amelioration of muscular status, results in enhanced motor functions; this suggests that preventing the accumulation of neurological lesions, that is, loss of MNS, could be considered as a first step in the development of a therapeutic strategy for SMA.

The effects of IPLEX observed in SMA animal models may have possible clinical implications: in a recent study, the combination of two compounds, an inhibitor of the proteasome and a histone deacetylase inhibitor, was demonstrated to be more effective compared with the administration of the individual drugs (57). In particular, the potential synergic effects of this drug with those that work on *SMN2* to enhance its transcription or exon 7 inclusion could elicit marked improvements in terms of phenotypic modifications.

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DISCLOSURES

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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