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EFFECT OF LOCALLY DELIVERED IGF-1 ON NERVE REGENERATION DURING AGING: AN EXPERIMENTAL STUDY IN RATS

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Abstract

Age is an important predictor of neuromuscular recovery after peripheral nerve injury. Insulin-like growth factor 1 (IGF-1) is a potent neurotrophic factor that is known to decline with increasing age. The purpose of this study was to determine if locally delivered IGF-1 would improve nerve regeneration and neuromuscular recovery in aged animals. Young and aged rats underwent nerve transection and repair with either saline or IGF-1 continuously delivered to the site of the nerve repair. After 3 months, nerve regeneration and neuromuscular junction morphology were assessed. In both young and aged animals, IGF-1 significantly improved axon number, diameter, and density. IGF-1 also significantly increased myelination and Schwann cell activity and preserved the morphology of the postsynaptic neuromuscular junction (NMJ). These results show that aged regenerating nerve is sensitive to IGF-1 treatment.

Keywords

aging; IGF-1; nerve injury; nerve regeneration; neuromuscular junction

Age is thought to be the most important predictor of neuromuscular recovery after peripheral nerve injury.¹ Of the 18 million extremity injuries recorded annually in the USA, >50% occur in patients 45 years of age or older.² Children often regain near-normal function after nerve injury,^{3,4} but adults or aged patients have slow or absent neuromuscular recovery. Over half of patients >50 years of age do not achieve any functional recovery following nerve repair,¹ resulting in impaired activities of daily living. Aging is known to affect the structure and function of the peripheral nerve,^{5,6} but the exact etiology of age-related impairment in regeneration is unknown, although delayed axonal regeneration^{7,8} and instability of the neuromuscular junction (NMJ) have been implicated.⁹

Insulin-like growth factor 1 (IGF-1) is a single-chain, 70-amino-acid polypeptide, with a molecular mass of 7.6 kDa, similar in structure to pro-insulin, and is highly conserved across species.¹⁰ IGF-1 plays a significant role in neuronal development, 11–12 recovery from

neuronal injury,^{13,14} neuronal survival,¹⁵ and neurite outgrowth following crush injury.¹⁶ In vitro studies have suggested that IGF-1 is produced locally by non-neuronal cells following injury and stimulates regeneration.¹⁷ Hansson et al. first showed that IGF-1 is secreted from Schwann cells in an autocrine fashion after peripheral nerve injury.¹³ IGF-1 has been shown to affect multiple facets of Schwann cell function in vitro, which likely contributes to improved nerve regeneration,¹⁸ including proliferation,¹⁹ mobilization, myelination,²⁰ and Schwann cell–axon interaction. IGF-1 knockout mice have defects in neurologic development²¹ in addition to impaired recovery from neuronal injuries.²² Thus, there is substantial evidence that IGF-1 plays a key role in neuromuscular recovery following injury.

Age and developmental stage are the major determinants of IGF-1 levels. IGF-1 levels peak during puberty, initiating and maintaining the pubertal growth spurt.²³ Following the pubertal growth spurt, IGF-1 levels decline²⁴ at a rate of approximately 14% per decade.^{25,26} With continued aging, IGF-1 concentrations fall to levels of 20–80% of values for young adults,²⁷ concordant with the decline in functional recovery following nerve transection and repair in aged adults.¹

Given the wide-ranging effects of IGF-1 on nerve regeneration and the known decline in IGF-1 with age, we sought to determine whether IGF-1 replacement would improve nerve regeneration in aged animals. Previously, we found that increasing systemic IGF-1 via pulsatile growth hormone injection did not improve nerve regeneration (unpublished data). Thus, the purpose of this study was to examine the effects of IGF-1 locally delivered to a nerve transection site in aged rats, and to evaluate neuromuscular recovery, axonal regeneration, myelination, Schwann cell activity, and the resulting changes at the NMJ. The hypothesis was that age-related decline in neuromuscular recovery following transection would be ameliorated by IGF-1 administered to the regenerating nerve.

METHODS

Animal Model

Thirty-two Fischer 344 × Brown Norway rats were obtained from the National Institute of Aging colony at the Harlan Sprague-Dawley facility (Indianapolis, Indiana). The animals were divided into two groups: young adult (8 months) and aged (24 months). Each age group was subdivided into saline and IGF-1 treatment groups. There were 6 young adult saline, 6 young adult IGF, 10 aged saline, and 10 aged IGF-1 animals. Animals underwent nerve transection and repair, as detailed in what follows, and were then killed after 12 weeks.

Surgical Procedure

Each animal was anesthetized with isoflurane, and an incision was made over the posterior aspect of the left thigh. Under the operating microscope, the sciatic nerve was exposed, and the tibial nerve was isolated and transected 1 cm from its insertion into the gastrocnemius. The nerve stumps were placed at opposing ends of a custom-made T-tube and the middle arm was attached to a minipump (Fig. 1). The minipump was buried subcutaneously under the skin of the back. The nerve conduit portion of the T-tube was made from 1016- μ m internal diameter, semiporous tubing (Micro-Renathane; Braintree Scientific, Braintree, Massachusetts), and the T-arm was constructed of Silastic tubing (Dow Corning, Midland, Michigan). An Alzet 2004 mini-osmotic pump (Durect Corp., Cupertino, California) delivered either normal saline or IGF-1 at a rate of 0.25 μ l/h. Half of the animals received recombinant human IGF-1 (Bachem AG, Torrance, California) at a 0.10- μ g/ μ l (100 μ g/ml) concentration. Thus, final IGF-1 delivery was 0.025 μ g/h, modeled after previous studies of IGF-1 infusion.²⁸ The other animals received normal saline only. The subcutaneous pumps

were replaced under anesthesia after 6 weeks. At 12 weeks after nerve transection and repair, tissue was harvested, and the animals were overdosed with isoflurane and perfused with fixative.

Animals were group-housed and allowed food and water ad libitum. All experiments were conducted under a protocol approved by the institutional animal care and use committee. The animals were housed in an appropriate facility in accordance with standards established by the Association for Assessment and Accreditation of Laboratory Animal Care.

Muscle and Tissue Harvest

At study termination, the animals were anesthetized with isoflurane, and the gastrocnemius was harvested. The muscle was cleaned, rinsed, flash frozen in liquid nitrogen, and stored at -80°C . Following removal of the gastrocnemius, the animals were transcardially perfused with warm saline followed by ice-cold 4% paraformaldehyde. The regenerated nerve was then removed and immersion-fixed in 2.5% glutaraldehyde.

Nerve Histomorphometry

The midpoint of the regenerated nerve was secondarily fixed in 1% osmium tetroxide, embedded in resin, sectioned to $1\text{ }\mu\text{m}$, and stained with toluidine blue. All images were acquired microscopically (AxioImager M1; Carl Zeiss, Jena, Germany) using AxioVision version 4.5 software. Images were imported into ImageJ (National Institutes of Health, Bethesda, Maryland) for analysis. For the peripheral nerve, the following measurements were taken: nerve diameter; nerve cross-sectional area; axon number; axon density (axons per mm^2); average axon diameter; and the average g-ratio (ratio of axon diameter to fiber diameter). Nerve diameter and nerve area were measured on $100\times$ or $200\times$ images. For axon number and axon density, two or three non-overlapping $400\times$ fields were obtained from each of the four quadrants of the nerve. For average axon diameter and the average g-ratio, three or four non-overlapping $1000\times$ fields were obtained from each of the four quadrants of the nerve. In each $400\times$ high-power field, the myelinated axons were counted, and the area sampled was calculated, yielding axon density. For axon diameter and g-ratio, a minimum of 100 nerve fibers were analyzed. In order to focus analysis on motor neurons, axons $<1\text{ }\mu\text{m}$ were not measured. For axon diameter, the measurement was made from the inner table of the myelin sheath and did not include the myelin layer. The g-ratio was calculated by measuring axon diameter and total fiber diameter.

mRNA Expression of GAP43

The lateral gastrocnemius was pulverized, and total RNA was extracted with Trizol (Sigma-Aldrich, St. Louis, Missouri). Quantity and purity of the RNA was assessed using a spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, Delaware), and quality of RNA was assessed by microcapillary gel electrophoresis (Model 2100 Bioanalyzer; Agilent Technologies, Santa Clara, California) prior to downstream applications. The mRNA was transcribed to cDNA using oligo-dT primers (Invitrogen, Carlsbad, California) and Superscript II (Invitrogen, Carlsbad, California). Primers were obtained from Applied Biosystems for GAP43 (NM_017195.3). Relative quantitative reverse transcript-polymerase chain reaction (qRT-PCR) was then performed (Model 7900HT; Applied Biosystems) with a 384-well block. Muscle creatine kinase (MCK) was used as an endogenous control, as it has been shown to be stably expressed in skeletal muscle with age.²⁹

Histology of Postsynaptic NMJs

The medial gastrocnemius was pinned to resting length and immersed in isopentane cooled with dry ice. The muscle was sectioned on a cryotome longitudinally in $25\text{-}\mu\text{m}$ sections. The

sections were permeabilized with 0.1% Triton X-100 for 20 minutes, and then treated with AlexaFluor 488–conjugated α -bungarotoxin (1:200; Invitrogen, Carlsbad, California) for 1 hour. Sections were rinsed and fixed in 1% paraformaldehyde for 1 hour. Confocal images were obtained on a laser scanning confocal microscope (Zeiss 510) with argon and helium–neon lasers. Slice thickness was set at $\leq 1.2 \mu\text{m}$. Slices were then merged to create the final image. The NMJs were then qualitatively evaluated.

Statistical Analysis

All data were imported into SigmaStat version 3.11 (Systat Software, Inc., San Jose, California) for analysis. For each outcome measure, a two-way analysis of variance (ANOVA) model was established with age and treatment as the independent variables. Post hoc pairwise comparisons were made with the Student–Newman–Keuls test. Transformations to ranked sums were made when the data were nonparametric. Type 1 error was set at 0.05.

RESULTS

IGF-1 Increases Axon Number, Diameter, and Density in Regenerated Nerve of both Young and Aged Animals

Examination of the peripheral nerve revealed that, in aged saline-treated nerves, there were fewer total axons, fewer axons per mm^2 , and less myelination than in young saline-treated nerves. IGF-1 had a marked effect on both young and aged animals, increasing axon number, diameter, and density, in addition to increasing myelination (Fig. 2). Quantitative data are presented in Figure 3. For the number of axons per nerve, IGF-1 increased total axon number in both young and aged animals; however, this was only statistically significant for aged animals. For axon density, IGF-1 increased axon density in both young and aged animals; however, this was only statistically significant for aged animals. For average axonal diameter, there was a significant increase in average axonal diameter in both young and aged animals when they were treated with IGF-1. There was no difference between the young IGF-1 group and aged IGF-1 group for number of axons per nerve, axon density, and average axonal diameter.

IGF-1 Increases Myelination and Schwann Cell Activity in Regenerated Nerve of both Young and Aged Animals

Three parameters of Schwann cell activity and myelination were examined: (1) myelin thickness; (2) axon/fiber ratio (g-ratio); and (3) GAP43 mRNA expression, a marker of Schwann cell activity.^{30,31} We found that IGF-1 significantly affected all measured aspects of Schwann cell activity and myelination (Fig. 4). For myelin thickness and g-ratio, this effect was not age-dependent. However, for GAP43 expression, aged animals had a greater response to IGF-1 than young animals (Fig. 4C).

IGF-1 Preserves Morphology of Postsynaptic NMJs in Aged Animals

The postsynaptic NMJs were stained with fluorescent-labeled α -bungarotoxin and qualitatively examined using laser scanning confocal microscopy (Fig. 5). In saline-treated young animals, the postsynaptic NMJs were highly contiguous, complex, and had deep gutters, similar to uninjured control NMJs (images not shown). In young IGF-1 animals, the NMJs were very similar to those in the young saline group. In the aged saline group, numerous NMJs displayed a loss of complexity, with markedly more shallow gutters, and a loss of perimeter and endplate area. This is consistent with the morphology of denervated NMJs.^{32,33} In contrast, in the aged IGF-1 animals, the complexity, gutter depth, and overall morphology more closely resembled that of young animals.

DISCUSSION

In this study we sought to determine whether IGF-1 would improve neuromuscular recovery after nerve injury during aging. We found that IGF-1 improved nerve regeneration by acting on the axons and Schwann cells, and secondarily on the NMJs. These results suggest that there is no loss of sensitivity to IGF-1 with age and that IGF-1 can improve regeneration after nerve injury during aging.

Previous studies have examined the effect of systemically administered IGF-1 during neuromuscular recovery in neonatal rats. Vergani et al. found that systemic IGF-1 promoted neuronal survival following crush injury and improved muscle reinnervation.³⁴ Kanje et al. found that locally delivered IGF-1 following crush injury in juveniles led to improved sensory function.³⁵ Tiancgo et al. found that IGF-1 locally delivered to an end-to-side repair improved muscle function in young rats.³⁶ To the authors' knowledge, the current study is the first to examine the effect of IGF-1 during nerve regeneration in aged animals.

Axonal outgrowth is central to nerve regeneration, and IGF-1 is a well-documented promoter of motor neuron survival,³⁷ axonal growth,³⁸ and axonal branching.¹⁸ In the regenerating nerve, IGF-1 is a key promoter of initial sprouting and subsequent elongation of axons³⁹ (for review, see Rabinovsky²). Although there has been extensive investigation of the effect of IGF-1 on neuron outgrowth and survival, nearly all of the studies were performed in vitro or in embryonic animals. Thus, it is not known how increased age affects the neuronal response to IGF-1. In this study we found that, for metrics of neuronal trophism, aged animals had a robust response to IGF-1 treatment, increasing axonal number, diameter, and density in the regenerated nerve segment. Thus, the neuronal response to IGF-1 of the regenerating axon does not appear to be limited due to age.

The Schwann cell is a key factor in promotion of nerve regeneration. The effect of IGF-1 on Schwann cells has also been studied. IGF-1 is known to promote Schwann cell survival,⁴⁰ motility,⁴¹ proliferation,⁴² and myelination.⁴³ However, the physiology of Schwann cells from aged animals is largely unknown. This study has addressed some of the parameters of Schwann cell function in vivo, particularly myelination and GAP43 expression. GAP43 is upregulated in nearly all myelinating Schwann cells of the distal stump following axotomy.³¹ Expression of GAP43 is postulated to play a role in the interaction between the regenerating axon and Schwann cell.³⁰ In this study, we found that GAP43 expression was significantly upregulated in both young and aged animals with IGF-1 treatment. In addition, GAP43 expression was higher in aged animals treated with IGF-1 than in similarly treated young animals, suggesting that age increases sensitivity to IGF-1 for this aspect of Schwann cell function. However, due to pooling of the whole muscle mRNA, in this study we could not differentiate between an increase in Schwann cell number and an increase in activity. Furthermore, because the sample was from muscle, the increase in GAP43 expression was due to intramuscular neurons, terminal Schwann cells, or both. GAP43 expression in the regenerated segment itself was not assayed. In addition to GAP43 activity, IGF-1 increased myelination in the regenerated nerve, independent of the increase in axon diameter. Thus, at the Schwann cell level, age does not impair the response to IGF-1 and results in an increased regenerative response.

Failure to achieve reinnervation of the muscle is thought to contribute to impaired neuromuscular recovery after nerve injury.⁴⁴ In this study we found by histologic examination that the motor endplate regained an innervated, pre-injury morphology in all but the aged saline group. This failure to reinnervate may have been due to delayed nerve regeneration or instability of the postsynaptic NMJ, both of which have been shown to occur with aging.⁹ In this study, treatment with IGF-1 may have accelerated axonal regeneration,

leading to earlier reinnervation of the muscle, and thus preservational of the postsynaptic NMJ.

One limitation of our study is the lack of functional and electrical assessments. Functional recovery is the gold standard for clinical outcomes in human subjects, but, historically, functional recovery in rodent models has been difficult to assess.⁴⁵ In rats, there is a poor correlation between functional, electrophysiologic, and histologic outcomes.⁴⁶ In this study, electrical measures of function were performed and showed a trend toward improvement with IGF-1 treatment, but this was not statistically significant (data not shown). Nonetheless, we were able to demonstrate that IGF-1 improved nerve regeneration in aged animals.

An important strength of our study is the in vivo comparison between young adult and aged rats after a complete nerve transection. Previous studies of IGF-1 on nerve regeneration utilized in vitro, neonatal, or juvenile animal models. In addition, many previous studies utilized crush injury models. Crush injuries in rodents typically have a near 100% rate of recovery and do not accurately model clinical scenarios wherein neuromuscular recovery is often very poor. Although previous studies have helped to answer many questions about the effects of IGF-1, this study is the first to examine in vivo the effects of age and IGF-1 on neuromuscular recovery following nerve transection.

Overall, our study has provided substantial evidence that locally delivered IGF-1 improves neuromuscular recovery after nerve injury in aged animals. We showed that, when local IGF-1 levels were equally supplemented in young and aged animals, nerve regeneration and NMJ preservation were similar, suggesting that IGF-1 may be a contributing factor in age-related impairment of neuromuscular recovery. However, our study has not conclusively demonstrated that IGF-1 deficiency is the immediate cause of age-related impairments in neuromuscular recovery after injury.

Abbreviations

ANOVA	analysis of variance
IGF-1	insulin-like growth factor 1
MCK	muscle creatine kinase
NMJ	neuromuscular junction
qRT-PCR	quantitative reverse transcript–polymerase chain reaction

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FIGURE 1.

Diagram of experimental setup. The tibial nerve was transected 1 cm from the insertion into the gastrocnemius and allowed to relax to create a 7-mm gap. A custom-made T-tube device was interposed between the nerve ends and secured with a 9-0 nylon suture. The middle arm of the T-tube was connected to an osmotic pump, which delivered either IGF-1 or normal saline at 0.25 $\mu\text{l/h}$.

FIGURE 2.

Cross-section of regenerated nerve segment. After 3 months, the regenerated nerve segment was removed, sectioned to 1 μm , stained with toluidine blue, and viewed at 1000 $\times$. (**A**) Young saline, (**B**) aged saline, (**C**) young IGF-1, and (**D**) aged IGF-1. Asterisk indicates representative myelinated axons. Note that each axon is surrounded by myelin of varying thickness. A myelinating Schwann cell (SC) can be seen in (**D**). In the saline-treated group, the young nerve had a larger number of axons and a greater axon density. With IGF-1 treatment, both groups had increases in axon number, diameter, and density (axons per mm^2). In addition, IGF-1 increased myelin thickness and myelin/fiber ratio (g-ratio). In the saline-treated groups, there are fewer myelinated axons, and the myelin is thinner (**A**, **B**). By histomorphometric analysis, however, the young saline-treated nerves (**A**) show a greater total number of axons and greater axon density (axons per mm^2). With IGF-1 treatment, both groups had increases in axon number, diameter, and density (axons per mm^2). In addition, IGF-1 increased myelin thickness and the myelin/fiber ratio (g-ratio). Scale bar = 10 μm . [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 3.

Effect of age and IGF-1 on axon number, density, and diameter. Quantification of histologic images reveals a significant effect of IGF-1 on (A) axons per nerve, (B) axon density (axons per mm²), and (C) average axonal diameter. Although there were differences due to age for axons per nerve and axon density, these were not statistically significant. Data are expressed as mean $\pm$ standard error * $P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 4.

Effect of age and IGF-1 on measures of myelination. Quantification of histologic images revealed a significant effect of IGF-1 on multiple facets of myelination and Schwann cell function. **(A)** Myelin thickness was significantly increased in IGF-1–treated nerves compared with saline-treated nerves. **(B)** Myelin thickness is known to increase with increases in axon diameter, and thus the g-ratio (axon: fiber diameter) was calculated. The significantly lower g-ratio in the IGF-1–treated groups indicates that the increases in myelination were independent of the increases in axon diameter. **(C)** GAP43 is a marker of Schwann cell activity and myelination. mRNA for GAP43 was assayed by RT-PCR. GAP43 expression in young and aged animals was not significantly different when both were treated with saline. GAP43 expression levels were found to be significantly increased by IGF-1 treatment in both young and aged animals compared with saline treatment. The GAP43 expression in aged IGF-1–treated animals was significantly higher than in young IGF-1 animals. Data are expressed as mean $\pm$ standard error. * $P < 0.05$ [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FIGURE 5.

Response of the neuromuscular junction to saline or IGF-1 treatment. (A) Young saline, (B) aged saline, (C) young IGF-1, and (D) aged IGF-1. The postsynaptic NMJ was stained with fluorescent-labeled α -bungarotoxin and imaged under a confocal microscope. In saline-treated young animals (A), postsynaptic NMJ was highly contiguous, complex, and had deep gutters. In aged saline-treated animals (B), there was a loss of complexity, with flattening of the gutters and a reduction in total postsynaptic area. This morphologic appearance is consistent with chronic denervation. In young IGF-1-treated animals (C), there were no differences when compared with saline-treated animals. In aged IGF-1-treated animals (D), there was an increase in the complexity and gutter depth, similar to the morphology seen in young animals. However, there was increased fragmentation seen in the aged IGF-1 group compared with the young IGF-1 or young saline groups. Scale bar = 20 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 1

Complete results of histomorphometry and GAP43 expression.

	Saline	IGF-1
Axons per nerve		
Young	6230 ± 746	11,359 ± 1392
Aged	2402 ± 1226	10,101 ± 3096
Average axon diameter (μm)		
Young	2.82 ± 0.16	3.53 ± 0.21
Aged	2.71 ± 0.18	3.59 ± 0.19
Axon density (axons per mm ²)		
Young	10,318 ± 2056	16,635 ± 1392
Aged	4506 ± 2298	17,767 ± 3865
Myelin thickness (μm)		
Young	0.35 ± 0.01	0.65 ± 0.09
Aged	0.37 ± 0.02	0.62 ± 0.07
Average g-ratio		
Young	0.80 ± 0.004	0.71 ± 0.006
Aged	0.79 ± 0.006	0.69 ± 0.01
GAP43 mRNA expression (RE)		
Young	0.80 ± 0.004	0.71 ± 0.006
Aged	0.79 ± 0.006	0.69 ± 0.01

Data expressed as mean ± SEM.