Peripheral Nerve Regeneration in Interleukin 6–Deficient Mice

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Background: Interleukin 6 (IL-6) is a multifunctional cytokine with effects on central and peripheral neurons.

Objective: To investigate the role of IL-6 in peripheral nerve regeneration by comparing IL-6 knockout and wild-type mice in a sciatic nerve model of injury and repair.

Design/Subjects: Forty C57/BL6 (wild-type) and 40 IL-6 knockout mice were randomly assigned to 1 of 4 groups: sham surgery, sciatic nerve crush injury, sciatic nerve transection without repair, and sciatic nerve transection with epineurial suture repair. Walking tracks were assessed preoperatively and postoperatively at 10-day intervals for 50 days by means of a previously described mouse sciatic functional index. Distal segments of the sciatic nerves were harvested at the completion of the study for histomorphometric evaluation.

Results: The wild-type and knockout mice that underwent sham surgery showed similarly unimpaired function (P = .64 on day 50). The IL-6 knockout mice with the crush injury demonstrated decreased function on day 10 compared with the wild-type mice (P < .01) but completely recovered by day 40 (P = .55). Both IL-6 knockout and wild-type mice that underwent nerve transection without repair failed to recover function (P = .06 on day 50). There was no statistical difference in recovery between wild-type and IL-6 knockout mice that underwent nerve transection with epineurial suture repair (P = .30 on day 50). The morphometric data showed no significant differences in distal axon count between the wild-type and knockout mice after suture repair or crush injury (P > .32).

Conclusions: The absence of IL-6 does not appear to impair peripheral nerve recovery after sciatic nerve injury. Although in vitro and in vivo studies suggest a role for IL-6 in peripheral nerve physiology, this cytokine does not appear to have a substantial effect on functional recovery in a mouse sciatic nerve injury and repair model.

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INTERLEUKIN 6 (IL-6) is a cytokine and therefore shares features common to the cytokine family, including a diversity of functions and a variety of sources. Interleukin 6 is synthesized by monocytes and macrophages, T cells, fibroblasts, endothelial cells, and astrocytes. Of interest, IL-6 is structurally homologous to the neurocytokines ciliary neurotrophic factor and leukemia inhibitory factor and also shares a common signal transducer molecule, gp130.

The studies of the physiological role of IL-6 in the central nervous system suggest a neurotrophic effect: IL-6 improves the survival of rat forebrain cholinergic neurons, rat midbrain cholinergic neurons, and rat spinal cord neurons in vitro. The expression of IL-6 messenger RNA (mRNA) and IL-6 receptor mRNA has been documented in several areas of the rat central nervous system.

Less is known about IL-6 involvement in the peripheral nervous system. Böhn et al first reported the presence of IL-6 in the peripheral nervous system by demonstrating its production by cultured Schwann cells. Whereas IL-6 mRNA expression has not been shown for intact nerves, it has been detected in degenerating mouse peripheral nerves, with rapidly degenerating nerves showing increased mRNA when compared with slower wallerian degeneration. Recently, a study in transgenic mice showed accelerated peripheral nerve regeneration in mice in which the expression of IL-6 and its receptors had been deliberately up-regulated. These findings implicate IL-6 in the neural degeneration and regeneration process.

Genetic engineering in animals has provided transgenic mice in which specific genes are either up-regulated or deleted (so-called knockout mice). An IL-6 knockout mouse has been created, allowing for the evaluation of the phenotypic effects of delinquent IL-6. The development of a mouse sciatic functional index (SFI) offers a quantitative assessment of functional recovery after nerve injury in mice. The present study was therefore initiated to delineate the effect of IL-6 on peripheral nerve regeneration by comparing IL-6 knockout mice with wild-type mice in a functional assessment of nerve recovery.
RESULTS

Seventy-nine of a total of 80 animals completed 50 days of walking track analysis. One IL-6 knockout mouse from the sham group died early in the study of unknown causes.

SHAM SURGERY

There were no statistically significant differences in the preoperative SFI values among the different groups (P > .34) (Figure 1 and Figure 2). As expected, there was no statistical difference between the wild-type and the knockout mice in the sham surgery at any of the time points (P > .12).

CRUSH SURGERY

The IL-6 knockout mice in the crush group demonstrated significant injury on postoperative day 10 as illustrated by the decline in mean SFI to −60 (P = .002) (Figure 1). By postoperative day 20, the knockout mice in the crush group had recovered sciotic function equivalent to that of the sham group (P = .43). In contrast, the wild-type mice in the crush group failed to show a significant postoperative injury (average SFI, −16) when compared with the wild-type sham group (P = .60) (Figure 2). Notably, the crush injury was delivered consistently, and the operator was blinded to the treatment group. When compared with the IL-6 knockout mice in the crush group, there were statistical differences at 3 time points: on postoperative day 10, when the knockout group expressed poor function after crush injury, and on postoperative days 20 and 30, when the knockout mice recovered function above that of the wild-type mice in the crush group (P < .04). By postoperative day 40, there were again no significant differences between the IL-6 knockout and wild-type mice in the crush group (Table).

TRANSECTION WITHOUT REPAIR

Both the wild-type and IL-6 knockout mice in the transection without repair groups exhibited poor function throughout the study, with mean SFI values ranging from −83 to −99, with no statistical differences between the 2 groups (Figures 1 and 2 and Table). Of note, 2 of 8 knockout mice and 1 wild-type mouse in the transection without repair group developed contractures of the paws between days 40 and 50.
Appropriate prints for measurement were chosen for clarity and consistency at a point when the mice were walking at a moderate pace. If necessary, animals were walked several times to achieve measurable prints. Toe spread and pawprint length were measured with a digitizing pad linked to a computer (Macintosh Performa 6115 CD; Apple Computers, Cupertino, Calif) equipped with NIH Image software (National Institutes of Health, available at http://rsb.info.nih.gov/nih-image). Sciatric functional indexes were calculated by means of the formula described previously by Inserrea et al.16

Contractures may occur during the period of recovery,17 especially in animals in which no repair is performed. Walking tracks in animals with contractures are impossible to measure and are therefore assigned a score of −100, representing maximal injury. Because it is possible to have an SFI score lower than −100, when indexes fell below this level, they were normalized to −100 to prevent a negative bias compared with the animals with contractures.

MORPHOMETRIC ANALYSIS

At the completion of the study, the animals were killed by lethal injection of pentobarbital sodium. Segments of sciatic nerves (which included 3 mm to each side of the repair site) were harvested from each mouse and fixed in buffered 10% formalin solution. Histological slides were made by cutting 0.6-mm sections and staining with Bielschowsky silver stain specific for nerve axons. The slides were examined with a light microscope (Nikon Alpha phot 2YS2; Technical Instruments, San Francisco, Calif) equipped with a single-chip color video camera (CCD No. 70-5110; JEDMED, St Louis, Mo) projected onto a color monitor (No. PM-1971A, NEC, Tokyo, Japan). The monitor was linked to a computer (Macintosh Performa 6115 CD; Apple Computers) loaded with a Frame Grabber Card (Model LG3; Scion Corp, Frederick, Md), which captures images from the video screen and digitizes the analog signal for editing on the computer. The Scion Image program (Scion Corp), an upgraded version of NIH Image, was used in conjunction with the Scion Frame Grabber Card for image analysis. All measurements, including axon count, axon diameter, and nerve area, were performed on the distal segments of the harvested nerves.

The distal nerve area was determined by manually outlining a digitized image of the nerve at ×4 magnification. The calibrated Scion Image program calculated the area of the outline. The axon count was determined from a digitized image of the nerve at ×40 magnification. The Scion Image program randomly selected 10 areas of 1290 µm² each within the distal nerve section. Counts were performed by manually marking each axon in succession. The axon count for the entire nerve was then calculated by multiplying the mean axon count per random area by the nerve cross-sectional area and dividing the product by 1290 µm². The nerve cross-sectional area (in square micrometers) was determined from the digitized image of the nerve at ×40 magnification. The mean axon diameter was determined from a digitized image of the nerve at ×100 magnification. Twenty axons were chosen randomly and outlined on the computer screen. The Scion Image program measured the area and calculated the diameter of the axons. From these measurements, the mean axon diameter of each nerve was calculated.

STATISTICAL ANALYSIS

Preoperative functional data from all 8 groups were evaluated by means of analysis of variance to determine if any significant differences existed between the groups at the start of the study. The t test was used to test significance in the comparison of different pairs of groups.

TRANSECTION WITH EPINEURIAL SUTURE REPAIR

In the groups that underwent transection followed by suture repair, both the wild-type and IL-6 knockout mice showed immediate postoperative functional deficit on day 10, with a mean SFI of −90 and −86, respectively (P = .38). The recovery reached a maximum SFI of −78 on day 20 in the wild-type group and a maximum SFI of −80 on day 30 in the IL-6 knockout group (Figures 1 and 2). Five of 12 wild-type and 3 of 12 IL-6 knockout mice developed contractures. Because these tracks are assigned a value of −100 (representing maximal loss of function), no further improvement was detectable in the mean SFIs for these groups.

There was no statistically significant difference in either the maximal (P = .84) or final (P = .30) functional recovery achieved when the wild-type and the IL-6 knockout mice were compared (Table).

HISTOMORPHOMETRIC ANALYSIS

The histological data for the crush and suture repair groups confirmed the neural regeneration that was detected with the functional analysis, with axon numbers, axonal diameters, and nerve areas nearing normal values. Although the suture-repaired nerves in the wild-type mice had higher values than those in the IL-6 knockout mice with respect to all 3 of these measures, none of these differences reached statistical significance (P > .22) (Figure 3). Likewise, no differences were noted in the crush surgical groups (P > .16).

Bolin et al13 demonstrated that IL-6 mRNA is up-regulated within the first 12 hours after nerve injury and suggested that the Schwann cell is the source of IL-6 production. Bourde et al21 similarly showed increased production of IL-6 very early after nerve injury, with levels of mRNA sharply decreasing after 24 hours. Interleukin 6, along with ciliary neurotrophic factor, is therefore one of the earliest proteins to appear after nerve injury. This chronological order led to its designation as a ‘lesion factor’ and initiated speculation that IL-6 has a role in a signal cascade of neural regeneration, as had previously been proposed for the central nervous system, in which IL-6 triggers nerve growth factor production.5,22

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Despite these previous findings implicating IL-6 in nerve regeneration,\textsuperscript{13,15,21} the results of the current study suggest that any role played by IL-6 in the functional outcome of peripheral nerve regeneration either is not critical or is not rate limiting, since the nerves of the genetically engineered animals incapable of producing IL-6 recovered function to levels comparable with that of the wild-type mice. The histomorphometric findings were consistent with the functional results. There were no statistically significant differences in axon count, axon diameter, or nerve area between the wild-type and knockout mice, suggesting again that IL-6 does not have a significant effect on nerve regeneration, at least by the 50th postoperative day.

It is possible that the evaluation techniques used in our study are not sufficiently sensitive to detect subtle alterations in the nerve regeneration process. In fact, the intervals of functional evaluation (10 days) may need to be shortened in future investigations to detect potentially rapid recovery of function, particularly in nerve crush models, since very early differences between the treatment groups would not have been detected in our model. Although our data appear to show minimal functional nerve injury in the wild-type mice after nerve crush, it is possible that the injury was sustained and then recovery achieved before the 10th postoperative day. Although not directly observed, it is possible that the wild-type mice achieved a more rapid recovery, potentially assisted by

\begin{table}
\centering
\begin{tabular}{|l|l|l|}
\hline
 & Wild-Type Mice & IL-6 Knockout Mice & P \\
\hline
Sham surgery & $-7.5 \pm 6.6$ & $-9.0 \pm 5.1$ & .64 \\
Crush surgery & $-13.4 \pm 12.9$ & $-6.7 \pm 3.9$ & .16 \\
Transection & \\
Without repair & $-98.6 \pm 2.9$ & $-93.1 \pm 7.6$ & .06 \\
With repair & $-93.4 \pm 10.1$ & $-85.7 \pm 20.6$ & .30 \\
\hline
\end{tabular}
\caption{Comparison of SFI Values at Day 50\textsuperscript{*}}
\end{table}

\textsuperscript{*All values are mean ± SD. SFI indicates sciatic functional index; IL-6, interleukin 6.}
the known early presence of IL-6. Since a standardized crush technique was used, it is difficult to conceive of any other systematic difference in injury between the wild-type and IL-6–deficient animals that received nerve crush. While the findings of some authors, particularly Hirota et al., provide compelling evidence of the value of up-regulated IL-6 in nerve regeneration, their findings are not necessarily inconsistent with our results. The present study depicts the effect of IL-6 deficiency on the functional aspects of nerve regeneration, an approach that has not previously been explored. Overexpression of genes producing some neurocytokines may enhance nerve regeneration, while deficiency of those same neurocytokines does not diminish regeneration, perhaps because other cytokines replace the function.

An additional consideration is the role of inflammation in nerve regeneration. Interleukin 6, a cytokine and well-known acute-phase reactant, is a key mediator of the inflammatory response. Furthermore, inflammation appears to support nerve regeneration. Therefore, it seems logical that increased levels of IL-6 would enhance nerve regeneration. Alternatively, while it has been shown that IL-6–deficient mice exhibit a defective inflammatory process, studies have not previously been undertaken to determine whether this deficiency, in the presence of sufficient quantities of other neurocytokines such as ciliary neurotrophic factor and leukemia inhibitory factor, impairs the functional outcome of nerve regeneration. Our results suggest that it does not; this seeming incongruence may similarly be explained by the possibility that increased inflammation enhances regeneration, while the effect of reduced inflammation is neutral.

CONCLUSIONS

Interleukin 6 does not appear to play a critical role in the overall process of peripheral nerve regeneration in the mouse. Although subtle, early consequences of the absence of IL-6 production would not have been detected in our model, the histomorphometric results confirm the functional findings that overall nerve recovery is not impaired with IL-6 deficiency.

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REFERENCES


