

# Accelerated Nerve Regeneration in Mice by Upregulated Expression of Interleukin (IL) 6 and IL-6 Receptor after Trauma

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## Summary

In this study we aimed to examine a role for interleukin 6 (IL-6) and its receptor (IL-6R) in peripheral nerve regeneration in vivo. We first observed that cultured mouse embryonic dorsal root ganglia exhibited dramatic neurite extension by simultaneous addition of IL-6 and soluble IL-6R (sIL-6R), a complex that is known to interact with and activate a signal transducing receptor component, gp130. After injury in the hypoglossal nerve in adult mice by ligation, immunoreactivity to IL-6 was upregulated in Schwann cells at the lesional site as well as in the cell bodies of hypoglossal neurons in the brain stem. In the latter, upregulation of the immunoreactivity to IL-6R was also observed. Regeneration of axotomized hypoglossal nerve in vivo was significantly retarded by the administration of anti-IL-6R antibody. Surprisingly, accelerated regeneration of the axotomized nerve was achieved in transgenic mice constitutively expressing both IL-6 and IL-6R, as compared with nontransgenic controls. These results suggest that the IL-6 signal may play an important role in nerve regeneration after trauma in vivo.

IL-6 is expressed in a wide variety of cell lineages including monocytes/macrophages (1), T cells (2), fibroblasts (3, 4), and endothelial cells (5, 6) in response to diverse stimuli. It is also of interest to note the presence of IL-6 in the nervous system because this molecule is structurally homologous to such neuro-acting cytokines as ciliary neurotrophic factor (CNTF)<sup>1</sup> and leukemia inhibitory factor (LIF) (7) which share, in their receptor complexes, the IL-6 signal transducer, gp130 (8–19). In the central nervous system, IL-6 expression was observed, for instance, in the pyramidal neurons of the hippocampus and granular cells of the cerebellum (20). Upregulation of IL-6 in the central nervous system after viral infection and mechanical crush was reported (21–23). Although it has been observed that IL-6 acts as a survival factor for nerve cells including rat mesencephalic and septal neurons in vitro (24, 25), its role in vivo has been demonstrated poorly. Whereas gp130 is widely expressed, the spectrum of the targets of IL-6 in the nervous system appears to be much narrower than that of LIF and CNTF due to relatively limited expression of

IL-6R in the nervous system. In this study, we focus on a role of IL-6 and IL-6R in peripheral nerve regeneration after trauma. We first show that peripheral nerve cells, which normally are not responsive to IL-6, have a potential to respond to this factor when IL-6R is simultaneously present. We also show that both IL-6 and IL-6R proteins are upregulated after nerve injury. With further experiments using blocking Ab to IL-6R and transgenic (TG) mice expressing IL-6 and IL-6R, we discuss the contribution of IL-6 and IL-6R to nerve regeneration in vivo.

## Materials and Methods

**Culture of Dorsal Root Ganglia.** Dorsal root ganglia (DRGs) from 17-d ICR-embryos were embedded in 0.1% collagen (Koken, Tokyo, Japan) in DME/Ham's12 (GIBCO BRL, Gaithersburg, MD), 10% FCS in multidishes (Nunc Inc., Naperville, IL) (1 DRG/0.3 ml/well). DME/Ham's12 with 10% FCS containing either human IL-6 (2 µg/ml), human soluble IL-6R (sIL-6R; 1 µg/ml), IL-6 plus sIL-6R (2 µg/ml and 1 µg/ml, respectively), or rat CNTF (2 µg/ml) was then overlaid (0.9 ml/well). DRGs were incubated for 4 d and photographed (Nikon, Tokyo, Japan).

**Nerve Injury and Immunostaining.** Under pentobarbital anesthesia, mice were positioned supine and right and left hypoglossal nerves were carefully exposed through a ventral neck incision and mobilized at a site proximal from the bifurcation of the nerve near the

<sup>1</sup> Abbreviations used in this paper: CNTF, ciliary neurotrophic factor; DRG, dorsal root ganglion; LIF, leukemia inhibitory factor; pAb, polyclonal antibody; sIL-GR, soluble IL-GR; TG, transgenic.

hyoid bone. The nerve on one side was then ligated with a piece of string. 1 wk after operation, the mice were anesthetized and perfused through the heart with 500 ml heparinized phosphate buffer (0.1 M, pH 7.4), followed by 750 ml of 4% paraformaldehyde in phosphate buffer. The ligated and contra-lateral sham-operated nerves and brain stem were removed and postfixed overnight with 2% paraformaldehyde and 0.2% picric acid in 0.2 M phosphate buffer (pH 7.4); they were then partially dehydrated overnight with 30% sucrose. Frozen sections (7  $\mu$ m) were placed on gelatin-coated glass slides, dried, and rehydrated for 5 min with 0.1 M phosphate buffer, pH 7.0, containing 1% Triton X-100. The sections were then blocked for 3 h with 0.1 M PBS containing 0.3% Triton X-100, 1% bovine serum albumin, and 1% normal goat serum (blocking buffer). The sections were incubated with polyclonal rabbit Ab to mouse IL-6 or mouse IL-6R (5  $\mu$ g/ml; kindly provided by Drs. Y. Koishihara and Y. Ohsugi, Chugai Pharmaceutical, Gotemba, Japan) in the blocking buffer overnight, and washed three times. The immunoreactivity was detected with affinity-purified biotinylated anti-rabbit Ig and Vectastain™ ABC Reagent (overnight incubations; Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's procedures.

**Assay for Nerve Regeneration.** Right and left hypoglossal nerves were carefully exposed as described above. The nerve was transected with a pair of scissors. The transected nerve was laid in a normal position, and the opening at the ventral neck was stitched. About 1  $\mu$ l of Fluorogold (Fluorochrome Inc., Englewood, CO) was injected into both sides of the tongue in a careful bilateral manner on the day of each assay. 2 d later, mice were killed and the brains were frozen in crushed dry ice. Fresh frozen sections were made, the tracer dye was visualized by fluorescence microscopy, (Nikon) and the number of Fluorogold-stained nerve cell bodies were counted. Regeneration rate was assessed by calculating the percent ratio of the number of stained cell bodies on the operated side of the brain stem to that in the sham-operated side.

**Treatment with Anti-IL-6R Ab.** Right and left hypoglossal nerves in the same animal were exposed as described above, and the nerve on one side was transected. Either anti-IL-6R mAb (26) or control IgG was intraperitoneally injected weekly five times. Regeneration rate was assessed using Fluorogold as described above.

**TG Mice.** Two TG lines expressing either human IL-6 or human IL-6R were separately prepared as reported previously (27). These two lines were mated to obtain double-TG mice expressing both human IL-6 and IL-6R proteins. The offspring were classified into four types by transmitted genes: 6<sup>+</sup>6R<sup>+</sup>TG, 6<sup>+</sup>6R<sup>-</sup>TG, 6<sup>-</sup>6R<sup>+</sup>TG, and 6<sup>-</sup>6R<sup>-</sup>TG, where 6<sup>+</sup> and 6R<sup>+</sup> represent the presence of IL-6 and IL-6R transgenes, respectively. In this study, we operated on 3-mo-old mice weighing ~30 g.

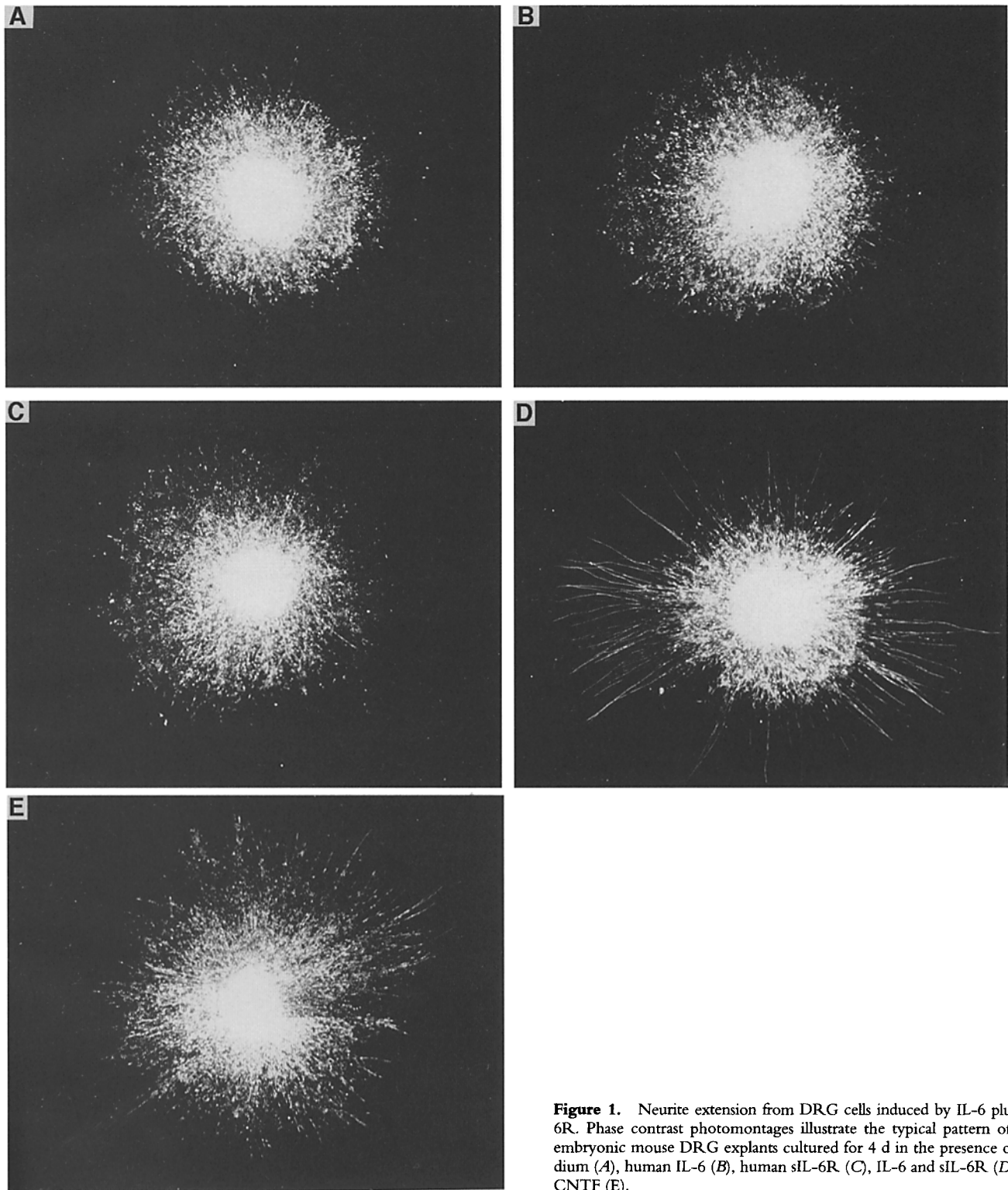
## Results

**Neurite Extension from DRG Cells Induced by IL-6 Plus sIL-6R.** A neurotrophic role of IL-6 signaling was first examined in cultured DRG from mouse embryos. In this experiment we used IL-6 and sIL-6R, a combination of which is known to interact with and activate gp130, as does the complex of IL-6 and membrane-anchored IL-6R (11). As shown in Fig. 1, simultaneous addition of IL-6 and sIL-6R induced significant neurite extension from DRG. This combination was as (Fig. 1 E) or even slightly more potent as CNTF. Neither IL-6 nor sIL-6R alone had any effect, presumably because DRG cells express gp130 but not IL-6 or IL-6R. In fact, immunoreactivity to gp130 but

not IL-6R was detectable in mouse embryonic DRG (Kiyama, H., K. Yoshida, and T. Taga, unpublished data). Neurite extension in this experiment was detectable on day 1 and most obvious on days 3 and 4. The result suggested that peripheral nerve cells have the potential to respond to the IL-6 signal to extend neurites, and implied that those cells might exhibit such response in vivo when IL-6 is produced locally and when the cells are induced to express IL-6R. This made us examine whether IL-6 and IL-6R are induced after nerve injury and are involved in nerve regeneration in vivo. In this line of study, we chose the mouse hypoglossal nerve for its ease of manipulation and its advantageous nature, that is, that each of the cell bodies localized in the brain stem extends the axon unilaterally towards the same side of the tongue.

**IL-6 Expression in the Injured Hypoglossal Nerve.** Both sides of the hypoglossal nerves were carefully exposed through a ventral neck incision, and only one of the two was ligated with strings (see Materials and Methods). 1 wk after surgery, the ligated and contra-lateral sham-operated nerves, as well as brain stem, were immunostained with rabbit anti-mouse IL-6 polyclonal antibody (pAb) or rabbit anti-mouse IL-6R pAb. As shown in Fig. 2, Schwann cells in the operated hypoglossal nerve at the position 500  $\mu$ m distal from the ligated site showed strongly enhanced staining for IL-6, as compared with the sham-operated nerve. The immunoreactivity to IL-6R was, unanticipatedly, only marginally upregulated in the ligated nerve (data not shown), probably because the level of IL-6R protein in the axon of the hypoglossal nerve was below the detection limit (with the use of this pAb).

**Immunolocalization of IL-6 and IL-6R in the Hypoglossal Nuclei in the Brain Stem.** We then performed immunohistochemical staining of the nerve cell bodies in the brain stem. As shown in Fig. 3, immunoreactive signals to IL-6 and IL-6R were both upregulated in the operated side of the medulla at the hypoglossal nucleus. A receptor-mediated mechanism of binding, uptake, and retrograde transport of neurotrophic factors such as nerve growth factor (NGF) and LIF have been demonstrated (28, 29). These factors bind to their receptors on the axon surface and the factor-receptor complexes are suggested to be internalized into endocytic vesicles, leaving the intracellular domains of the transmembrane receptors free to interact with cytoplasmic signaling molecules. This topology is considered to allow the activated receptor to form complexes with signaling molecules in the course of retrograde transport, either in the axon or in the cell body. Such a consideration raises the possibility that, via a similar mechanism, IL-6 protein produced at the lesion site binds to IL-6R expressed on the axon surface, the IL-6/IL-6R complex is then transferred to, and accumulated in, the cell bodies of neurons, allowing these proteins to be detectable in the brain stem by immunostaining. It was postulated that a relatively low level of IL-6R expression led to the low intensity of IL-6 staining in the cell body. These results suggested that IL-6 is produced after nerve injury in Schwann cells and used by the surviving nerve cell bodies through the remaining axons, via the

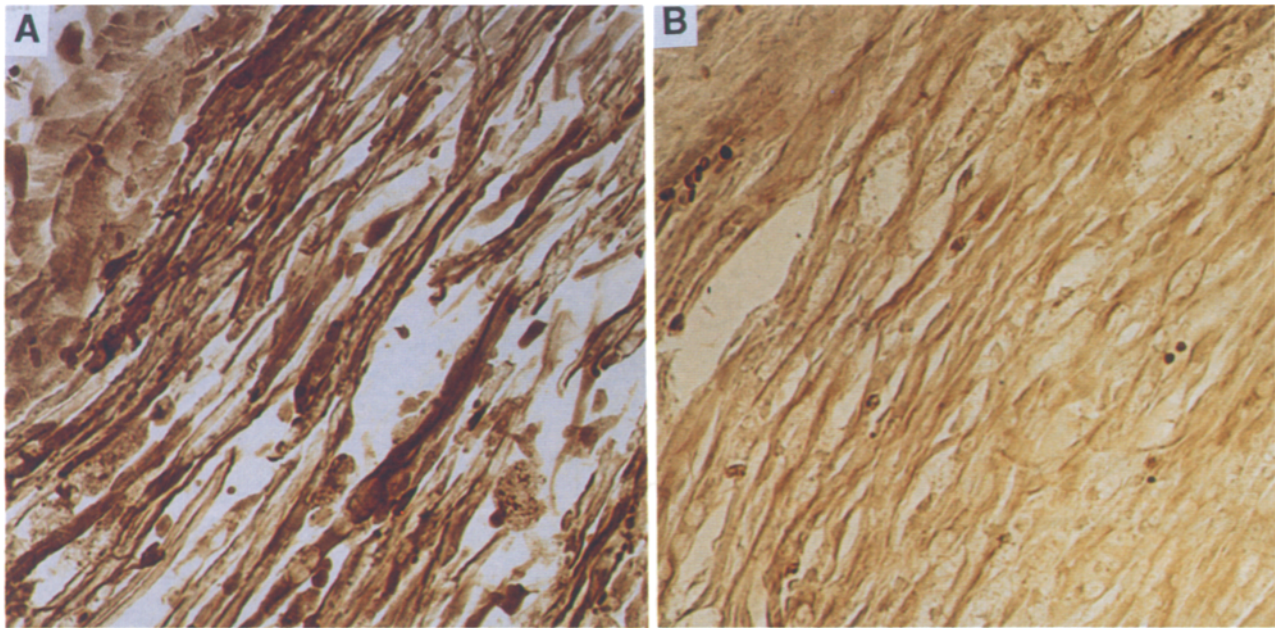


**Figure 1.** Neurite extension from DRG cells induced by IL-6 plus sIL-6R. Phase contrast photomontages illustrate the typical pattern of 17-d embryonic mouse DRG explants cultured for 4 d in the presence of medium (A), human IL-6 (B), human sIL-6R (C), IL-6 and sIL-6R (D), and CNTF (E).

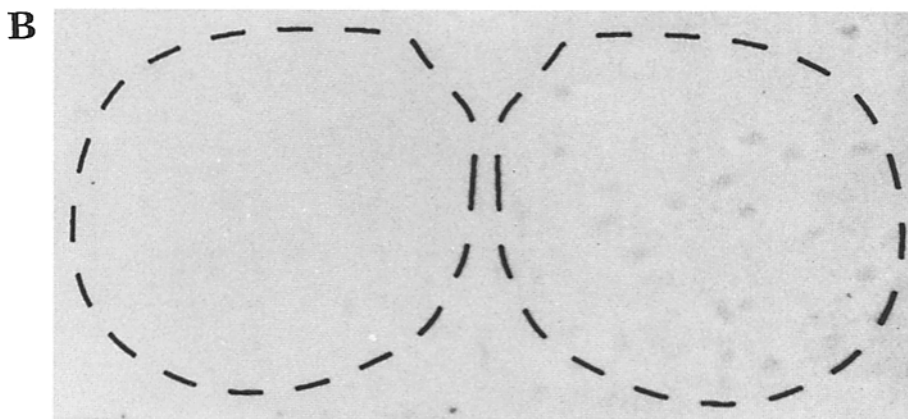
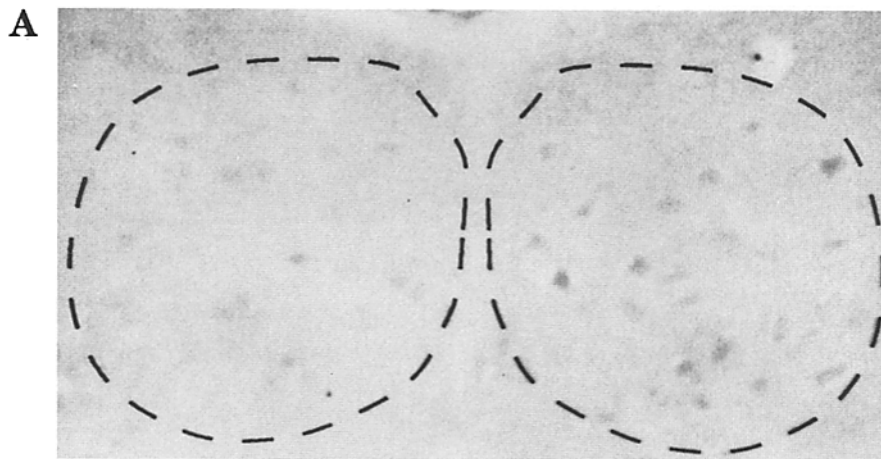
upregulated IL-6R protein, most likely to support regeneration of axons.

*Retardation of Regeneration of the Transected Hypoglossal Nerve by Administration of Anti-IL-6R Ab in vivo.* To ascertain whether the injury-induced upregulation of IL-6 and

IL-6R is involved in nerve regeneration in vivo, anti-mouse IL-6R mAb which blocks IL-6 binding to the receptor was administered after axotomy (see Materials and Methods). In normal IgG-treated mice, the number of Fluorogold-positive cell bodies in the operated side of the hypoglossal

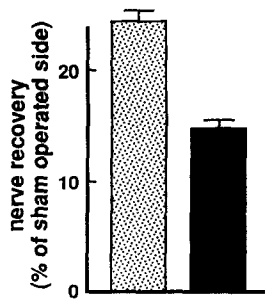


**Figure 2.** Upregulation of IL-6 expression in the injured hypoglossal nerve. (A) Anti-IL-6 staining of the nerve 1 wk after ligation injury at position 500  $\mu$ m distal from the ligation site. (B) Sham-operated nerve at position equivalent to that in A.



**Figure 3.** Immunolocalization of IL-6 and IL-6R in the hypoglossal nuclei in the brain stem. (A) Anti-IL-6R staining. (B) Anti-IL-6 staining. (Right) Operated side; (left) sham-operated side.





**Figure 4.** Retardation of regeneration of the transected hypoglossal nerve by anti-IL-6R Ab. Hypoglossal nerves were transected and the operated mice ( $n = 3$ ) were intraperitoneally administered with control rat IgG (stippled column) or rat anti-mouse IL-6R blocking Ab (filled column) five times with 1-wk intervals (starting on the seventh day after operation). The regeneration rate was calculated as described in the text. Vertical bars, SD.

nucleus was  $23.0 \pm 0.90\%$  of that in the sham-operated side. In contrast, in the anti-IL-6R mAb-treated mice, the number of Fluorogold-positive cells in the operated side was only  $14.8 \pm 0.76\%$  of that in the sham-operated side (Fig. 4). The results indicated that the administration of anti-IL-6R blocking mAb reduced the regeneration of the nerve by 36% when assayed 5 wk after surgery. This suggests that the IL-6 signal is required to a substantial extent for peripheral nerve regeneration.

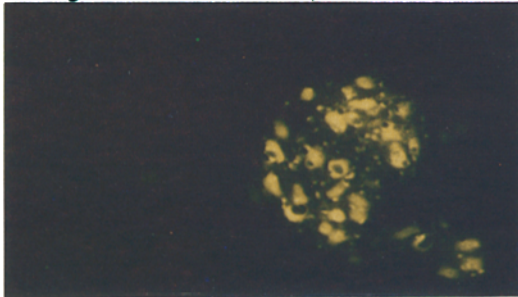
**Accelerated Nerve Regeneration in TG Mice Constitutively Expressing both IL-6 and IL-6R.** We then examined whether the rate of nerve regeneration is affected in TG mice overexpressing human IL-6 and/or human IL-6R. In the regeneration assay described above, Fluorogold-positive cells in the operated side of the hypoglossal nucleus in the brain stem 4 wk after axotomy were not so prominent in the non-TG control (see Fig. 5A for a representative section: the number of tracer positive cells was  $14.2 \pm 2.1\%$  of that

in the sham-operated side [the average of the results from three mice]). In contrast,  $6^{+}6R^{+}TG$  mice showed apparently more improved regeneration: the number of Fluorogold-positive cells in the operated side was  $40 \pm 3.0\%$  (the average of the results from three mice) of that in the sham-operated side (i.e., a 2.8-fold increase from the control; see Fig. 5B for a representative section). At this stage, nerve regeneration in mice carrying either of the transgenes ( $6^{+}6R^{-}TG$  and  $6^{-}6R^{+}TG$ ) was comparable with that in  $6^{-}6R^{-}TG$  mice. The kinetics of nerve regeneration was measured in the TG mice of the four different genotypes. As shown in Fig. 6, the  $6^{+}6R^{+}TG$  mice achieved nerve regeneration 1.5–2.0 wk faster than the other TG mice. These results support the idea that IL-6 signaling contributes to nerve regeneration.

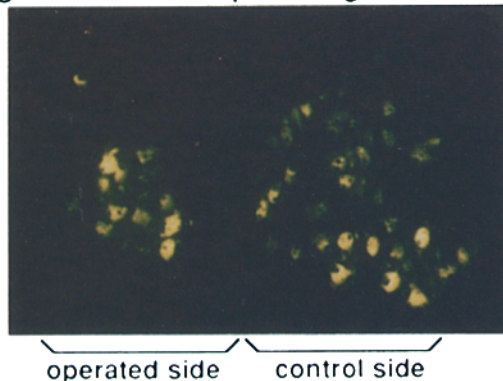
## Discussion

Administration of CNTF, a member of the IL-6 family of cytokines, is known to: (a) prevent degeneration of axotomized facial motor neurons in neonatal rats; (b) attenuate motor deficits in *pmn/pmn* and *wobbler* mice with neuromuscular dysfunction; and (c) exert myotrophic effects by attenuating morphological and functional changes associated with denervation of rat skeletal muscle (30–33). In the intact nerve, CNTF immunoreactivity is observed predominantly in the cytoplasm of myelin-associated Schwann cells. After injury, significant quantities of CNTF protein are extracellularly released from the Schwann cells, predominantly at a distal area from the lesion (34–37). Thus, it seems that

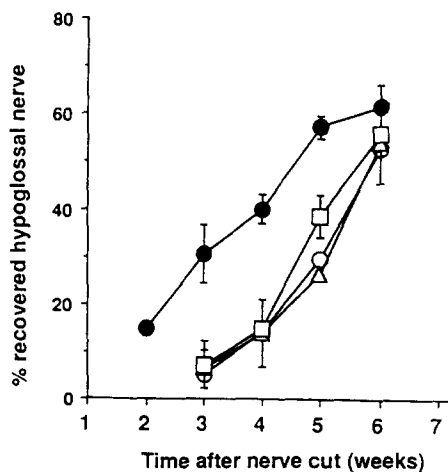
### (A) Non-transgenic littermate (4 weeks after operation)



### (B) Transgenic mouse expressing hIL-6 and hIL-6R



**Figure 5.** Accelerated regeneration of the transected hypoglossal nerve by constitutive expression of IL-6 and IL-6R. Fluorogold staining of the hypoglossal nuclei of the non-TG control (A) and TG mice expressing both IL-6 and IL-6R (B) prepared 4 wk after axotomy are shown. (Right) Operated side; (left) sham-operated side.



**Figure 6.** Time course of nerve regeneration after axotomy in control and TG mice expressing IL-6 and/or IL-6R. In this assay, 6<sup>+</sup>6R<sup>+</sup>TG (*n* = 18; closed circles), 6<sup>+</sup>6R<sup>-</sup>TG (*n* = 15; open squares), 6<sup>-</sup>6R<sup>+</sup>TG (*n* = 15; open triangles), and 6<sup>-</sup>6R<sup>-</sup>TG mice (*n* = 15; open circles) were used. Hypoglossal nerves were transected and nerve regeneration was examined at the time points indicated. Each dot represents the average value from three individual mice. Vertical bars, SD.

CNTF acts as a lesion factor similarly as has been observed for IL-6 in the present study. However, the regulation of the protein amount of IL-6 before and after injury appeared to be different from that of CNTF; IL-6 immunoreactivity was not detectable in the intact nerve, but was elevated only after injury due to the upregulated synthesis of the IL-6 protein that is likely to become available for the neurons being regenerated. Thus, CNTF appears to work immediately upon injury and IL-6 may follow CNTF after de novo synthesis. Concerning the expression of IL-6 in the peripheral nerve, previous reports (38, 39) have shown that the IL-6 transcripts, as detected by Northern blotting, were upregulated in the injured sciatic nerve and facial nerve tissues. In these studies, however, functional importance of the IL-6 mRNA elevation, histological localization of the IL-6 protein, and IL-6R upregulation have not been demonstrated. In our present study, immunoreactivity to IL-6 was upregulated in Schwann cells at the lesion site as well as in the hypoglossal nerve cell bodies in the brain stem. In the latter, upregulation of the immunoreactivity to IL-6R was also obvious.

A functional receptor complex for CNTF is composed of three different chains, i.e., CNTFR, LIFR, and gp130 (8–19, 40). The CNTF signal is transmitted through the membrane via the LIFR/gp130 heterodimer which is formed by binding of CNTF to CNTFR. In the previous study, we have shown that the simultaneous addition of IL-6 and sIL-6R,

which induces the gp130/gp130 homodimer, mimics CNTF (41). In the present study, however, the biological meaning of the simultaneous expression of IL-6 and IL-6R in nerve-injured mice and 6<sup>+</sup>6R<sup>+</sup>TG mice does not seem to be a simple mimicry of the biological function of CNTF, but instead appears to be a contribution to nerve regeneration. This is because both IL-6 and IL-6R protein are upregulated after nerve injury and, more importantly, anti-IL-6R Ab retards the regeneration of transected axons. The functional aspect of nerve regeneration by IL-6 signaling remains to be tested.

TG mice expressing human IL-6R alone (6<sup>-</sup>6R<sup>+</sup>TG) did not exhibit faster nerve recovery, because endogenously expressed mouse IL-6 does not bind to human IL-6R (42). At the fifth week of the experiment (Fig. 6), 6<sup>+</sup>6R<sup>-</sup>TG mice expressing human IL-6 alone showed slightly higher regeneration, but the extent was still very much smaller than that in 6<sup>+</sup>6R<sup>+</sup>TG mice. All in all, the 6<sup>+</sup>6R<sup>-</sup>TG mice did not show significant acceleration in nerve recovery. Since human IL-6 is capable of binding to human and mouse IL-6R (42), this phenomenon could be explained if we were to assume that the amount of mouse IL-6 expressed after nerve injury is already sufficient for that of mouse IL-6R.

It might be worth noting the number of neurons in the hypoglossal nucleus in the brain stem of the 6<sup>+</sup>6R<sup>+</sup>TG mice. We counted the number of Fluorogold-stained cell bodies in the hypoglossal nucleus in the brain stem of non-operated TG mice that had been injected with Fluorogold into the tongue. It was not significantly increased in the 6<sup>+</sup>6R<sup>+</sup>TG mice (on average, 27.3 ± 1.2/section in the 6<sup>+</sup>6R<sup>+</sup>TG and 25.0 ± 2.8/section in 6<sup>-</sup>6R<sup>-</sup>TG mice). Since we have not determined the amount of transgene-derived human IL-6 and human IL-6R during embryogenesis and early postnatal stages, we could not conclude whether the simultaneous expression of the IL-6 and IL-6R proteins developmentally affect the number of hypoglossal neurons.

gp130-stimulatory cytokines other than IL-6, such as LIF, IL-11, and oncostatin M, deserve a thorough evaluation as additional potential neurotrophic molecules for the peripheral nerve. Indeed, in vitro and in vivo trophic effects of, for instance, LIF on motor neurons have been observed (43). The question as to whether individual gp130-stimulatory cytokines function redundantly or whether these factors act to perform distinct trophic actions in the peripheral nervous system still remains to be proved. It will be interesting to elucidate their physiological role during functional recovery of the neuron activities, their possible pathophysiological importance, and, as a consequence, their suitability for the treatment of nerve degenerative disease.

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