Major changes in the expression of the mRNAs for cholinergic differentiation factor/leukemia inhibitory factor and its receptor after injury to adult peripheral nerves and ganglia

(neurotrophic/neuropoietic cytokine/interleukin 6)

LISA R. BANNER AND PAUL H. PATTERSON

Biology Division, California Institute of Technology, Pasadena, CA 91125

Communicated by Seymour Benzer, April 22, 1994

ABSTRACT The neuroepoietic cytokine cholinergic differentiation factor/leukemia inhibitory factor (CDF/LIF) acts as a trophic factor, enhancing neuronal survival, and as a differentiation factor, altering neuronal gene expression. There is also evidence that it plays a role in the response of adult neural tissue to injury. We have examined this possibility further in rats by analyzing changes in the levels of mRNAs for CDF/LIF and its two receptor subunits in response to peripheral nerve damage in culture. Using a quantitative reverse transcription–PCR protection assay, we find that CDF/LIF mRNA increases dramatically (176-fold) in adult, but not neonatal, sympathetic ganglia and in adult dorsal root ganglia and sciatic nerve after organ culture for 24 hr. This mRNA is clearly detectable by in situ hybridization only in the nonneuronal cells of these structures. When the sciatic nerve is transected in vivo, CDF/LIF mRNA increases significantly in the region immediately proximal and distal to the lesion site. The mRNA for the ligand binding subunit of the CDF/LIF receptor complex decreases somewhat upon culture and nerve section. The dramatic rise in CDF/LIF mRNA after nerve injury is further evidence that this cytokine is involved in the response to damage, a function that overlaps with its postulated role in wounding or infection in several nonneuronal tissues.

Mammalian peripheral nerves can regenerate after injury. They also participate in the response to damage of other tissues, such as the inflammatory reaction in arthritis (1). Both the response to nerve injury and the generation of an inflammatory reaction involve a complex cascade of signals among neuronal axons, glia, and cells of the immune system. Much of this communication is mediated by cytokines/interleukins (ILs) and by neurotransmitters and neuropeptides released by neurons and possibly the immune cells. Prime candidates for involvement in these events are the neuroepoietic cytokines, an emerging family of proteins grouped not by extensive sequence identity but rather by redundancies in biological effects on cells of the nervous and hematopoietic systems, by the sharing of receptor subunits, and by predicted secondary structures (2–4). Members of this family recognized thus far include cholinergic differentiation factor/leukemia inhibitory factor (CDF/LIF), ciliary neurotrophic factor (CNTF), oncostatin M, growth promoting activity, IL-6, and IL-11 (4). All of these cytokines can regulate gene expression in peripheral neurons, and four of them have also been shown to exert trophic effects on various types of peripheral and central neurons (for review, see ref. 5).

A striking feature of the neuropeptides induced in cultured neonatal sympathetic neurons by CDF/LIF, CNTF, oncostatin M, and growth promoting factor is that several of these same neuropeptides are also induced in adult sympathetic neurons when the ganglia are damaged by explantation in short-term culture or by axotomy in vivo. For instance, substance P and vasoactive intestinal polypeptide (VIP) are induced both by nerve damage and by application of these neuroepoietic cytokines to pure neuronal cultures (4, 6, 7). In response to peripheral nerve damage, VIP is induced in dorsal root ganglia (DRGs) as well (8, 9). Moreover, CDF/LIF elevates VIP in dissociated DRG neurons (10). Part of the in vivo response to injury could be mediated by nonneuronal cells in the ganglia, since these cells have been shown to release CDF/LIF (11, 12). CDF/LIF involvement in the neuropeptide response to nerve damage is further indicated by results from mice in which the CDF/LIF gene was disrupted by homologous recombination. Ganglia from such mutant mice display a much reduced neuropeptide response to culturing or axotomy (13).

To further investigate the role of CDF/LIF in the events surrounding nerve damage, we have determined whether the levels of CDF/LIF and its receptor subunits change after nerve and ganglion injury. The major sources of CDF/LIF mRNA under these conditions were also localized by in situ hybridization. Some of these findings have been reported (14–16).

MATERIALS AND METHODS

Surgical Procedures and Organ Culture. Adult male and female rats were anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutol, Abbott; 40 mg/kg). The sciatic nerve was exposed and transected. To ensure that regeneration did not occur, a 3-mm piece of the nerve was removed and the remaining ends were deflected. Care was taken so that surrounding muscles were not damaged. The wound was closed with clips. Each time point is composed of data from nerves of nine animals. At the appropriate time, animals were killed by CO2 inhalation and various regions of the sciatic nerve were dissected and frozen at –70°C. Corresponding regions of nerve were removed from the contralateral side of each rat as control.

Fifteen superior cervical ganglia (SCGs) from postnatal day 1 (PI) rats and 15 adult DRGs and 10 SCGs from adult Sprague–Dawley (Simonson Laboratories, Gilroy, CA) rats were dissected, desheathed, and placed in organ culture. Ganglia were maintained in L15/C02 medium (17) supplemented with 5% (vol/vol) rat serum and nerve growth factor (NGF; 100 ng/ml) for 24 hr. Sciatic nerves from three adult animals were dissected, cut into sections ~1 cm long, and

Abbreviations: CNTF, ciliary neurotrophic factor; CDF/LIF, cholinergic differentiation factor/leukemia inhibitory factor; DRG, dorsal root ganglion; GAPDH, glyceraldehyde phosphate dehydrogenase; GPA, gp130, glycoprotein 130; LIFR, LIF receptor; NGF, nerve growth factor; P1 postnatal day 1; SCG, superior cervical ganglion; IL, interleukin; VIP, vasoactive intestinal polypeptide.
maintained under the same conditions as the ganglia. After culture, tissues were frozen at −70°C prior to RNA extraction. Each of the above experiments was performed twice. As controls, equal numbers of ganglia and nerves were dissected and frozen immediately at −70°C.

**RNA Extraction and RNase Protection Assay.** Total RNA was extracted from the tissues by the acid-phenol method (18). RNA was resuspended in distilled H2O and stored at −70°C. RNase protection analysis was performed as described (19). A portion of LIF receptor (LIFR), glycoprotein 130 (gp130), and glycolaldehyde phosphate dehydrogenase (GAPDH) cDNAs from rat kidney were isolated by the polymerase chain reaction (PCR). The PCR products were ligated into Bluescript KS (Stratagene) linear range mined that the values are expressed in arbitrary units. Control RNase protection experiments with known amounts of in vitro-transcribed sense RNA probes determined that the values obtained for each scan are within the linear range of the PhosphorImager. GAPDH levels measured before and after nerve transection revealed that the mRNA for GAPDH did not change significantly after transection.

**In Situ Hybridization.** Antisense and sense digoxigenin-labeled CDF/LIF probes were generated using the Ambion (Austin, TX) MEGAScript in vitro transcription kit. **In situ** hybridization was performed as described (20). Briefly, 20-μm sections of fixed cultured ganglia and nerve were hybridized overnight at 50°C with sense or antisense probes, either probe at 1–2 μg/ml. After RNase digestion, sections were incubated with an anti-digoxigenin antibody (Boehringer Mannheim) and the alkaline phosphate reaction product was visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

**RESULTS**

**Changes in the mRNAs for CDF/LIF and Its Receptor Subunits After Organ Culture of the SCGs.** To determine whether CDF/LIF mRNA changes in sympathetic ganglia in response to injury, neonatal (P1) and adult SCGs were placed in organ culture. After 24 hr, CDF/LIF mRNA was analyzed using a quantitative RNase protection assay. To standardize the CDF/LIF mRNA values, they were expressed as a ratio to GAPDH, an internal control for total RNA. This ratio also controls for variability in RNA recovery from the tissues, and since the GAPDH sample is quantified in the same sample and gel lane, it controls for variability in processing of the samples. Moreover, GAPDH levels were found not to be altered by nerve damage (see below). There was a 10-fold induction in the expression of CDF/LIF mRNA relative to GAPDH mRNA when P1 ganglia were cultured for 24 hr (Fig. 1A). CDF/LIF mRNA levels increased even more dramatically, however, when adult SCGs were placed in organ culture for 24 hr; the increase was 176-fold over levels in uncultured ganglia (Fig. 1A). The enormous increase in CDF/LIF mRNA after organ culture of adult ganglia was not paralleled by similar changes in its receptor mRNA expression. The levels of the ligand binding receptor subunit, LIFR, decreased by half in both cultured P1 and adult ganglia when compared to control values (Fig. 1B).

**Expression of CDF/LIF mRNA in Sensory Ganglia and Peripheral Nerve After Organ Culture.** As outlined above, motor and sensory neurons are also known to up-regulate certain neuropeptides after injury. To determine whether CDF/LIF mRNA levels change in response to sensory ganglion injury, adult DRGs were cultured for 24 hr and expression of CDF/LIF mRNA was monitored. As is the case for adult sympathetic ganglia, relatively low levels of CDF/LIF mRNA were present in normal adult sensory ganglia. After culture for 24 hr, however, the levels increased 43-fold (Fig. 2A), indicating that the CDF/LIF induction is not unique to the SCG. LIFR mRNA also showed a response similar to that of the SCG (Fig. 2B), decreasing slightly after culture, whereas gp130 mRNA levels remained constant. It is important to note that GAPDH levels measured before and after nerve transection revealed that the mRNA for GAPDH did not change significantly after transection (data not shown).

The large difference in the inducibility of CDF/LIF between P1 and adult sympathetic ganglia (Fig. 1) could be due to the increase in the number of glial cells that occurs during postnatal development. To examine the CDF/LIF response in a tissue composed primarily of glial cells, we examined the effects of organ culture on adult sciatic nerve. In response to 24 hr of organ culture, the expression of CDF/LIF mRNA increased 76-fold over normal adult nerve (Fig. 3A). LIFR
mRNA decreased 4-fold after organ culture, whereas gp130 levels remained constant (Fig. 3B).

**CFL/LIF mRNA in Adult Sciatic Nerve Is Up-Regulated in Response to Transection in Vivo.** The pronounced increase in CFL/LIF mRNA expression in sciatic nerve after 24 hr of culture led us to investigate whether CFL/LIF might be regulated in a similar manner in vivo. Adult rat sciatic nerves were transected at mid-thigh level and CFL/LIF mRNA from different portions of the nerve was monitored from 24 hr to 14 days after transection. Three regions—immediately distal to the transection site (distal), immediately proximal to the transection site (proximal-1), and a second proximal portion, adjacent to proximal-1 but closer to the spinal cord (proximal-2), each ~1 cm long—were examined by RNase protection analysis. As with cultured sciatic nerve, there was an increase in CFL/LIF expression, although the in vivo increase was not as dramatic as in organ culture. Twenty-four hours after transection, the mRNA for CFL/LIF was ~11-fold higher in the region distal to the transection site and 13-fold higher in the proximal-1 region, when compared to their respective undisturbed contralateral regions (Fig. 4). After 5 days the expression of CFL/LIF in the proximal-1 region remained higher than the contralateral sites. By 14 days after injury, the level of CFL/ LIF in the proximal-1 region decreased while levels in the and proximal-2 regions remained essentially the same as those seen at 7 days. CFL/LIF mRNA levels in the proximal-2 region remained relatively unchanged throughout the 2-week period.

**The Ligand Binding Receptor Subunit for CFL/LIF Decreases in Response to Sciatic Nerve Transection.** Consistent with the results seen when ganglia and nerve were cultured for 24 hr (Figs. 1–3), CFLIR levels in the region immediately proximal and distal to the lesion site begin to decrease 24 hr after transection (Fig. 5). This decrease continued until day 5 in the proximal-1 region after which time the expression returned to and exceeded those of the control values. Distal to the transection site, CFLIR mRNA levels were depressed relative to control values throughout the entire 2-week period studied. The levels of CFLIR mRNA in the proximal-2 region did not change relative to control values at any times examined (data not shown). There was a slight increase in the expression of gp130 in the distal and proximal-1 regions but the values did not differ significantly from those of the contralateral regions (data not shown).

**CFL/LIF Is Expressed in the Nonneuronal Cells of Peripheral Ganglia and Nerve.** While it has been known for many years that CFL/LIF is produced by cultured nonneuronal cells from ganglia, the identity of the cell type(s) responsible has not been determined (11). Both fibroblasts and Schwann cells can, however, secrete CFLIR in dissociated cell culture (14, 21, 22). Because the mRNA for CFLIR is present at very low levels in normal ganglia and nerve, our attempts to determine which cells express it were unsuccessful. Since the mRNA is greatly increased in response to organ culture, however, we were able to observe cells strongly positive for CFLIR mRNA by performing in situ hybridization on ganglia and sciatic nerve that had been cultured for 24 hr. The results of using digoxigenin-labeled antisense CFLIR probes to hybridize sections of adult SCGs and sciatic nerve are illustrated in Fig. 6. In ganglia (Fig. 6A), the staining was in small cells and not neurons. In sciatic nerve (Fig. 6B), staining was in elongated cells, most of which appear to have processes on one or both ends; these morphologies closely resemble Schwann cells. While the staining is clearly nonneuronal in the ganglia, the precise identity of the labeled cells is not certain. To determine whether the CFLIR-positive cells were macrophages, we stained ganglia and nerve with macrophage-specific antibodies in conjunction with in situ hybridization for CFL/LIF mRNA. The cells positive for CFLIR mRNA did not colocalize with the macrophage-specific antibodies MUC-102 or OX-42 (data not shown).

**DISCUSSION**

The ability of the body to respond quickly and effectively to damage is a crucial step in the recovery process, and the release of cytokines by a variety of cell types plays a critical role in the initial response to injury. We find that a member of the hematopoietic cytokine family, active in both the hematopoietic and nervous system, is dramatically up-regulated in response to peripheral nerve damage. The increase in CFLIR mRNA expression in the adult SCGs and DRGs is consistent in time course and magnitude with the changes in neuropeptide expression found in the ganglia after injury. The lower CFLIR response in neonatal ganglia suggests that the cell type that expresses it may be present in fewer numbers or is less responsive in the neonate. The fact...
that we find CDF/LIF expressed in nonneuronal cells of ganglia and nerve is consistent with the fact that P1 ganglia contain many fewer glial cells than adult ganglia.

CDF/LIF increases the survival of embryonic sensory neurons in vitro (23) and was recently shown to be present in vivo (24). This cytokine can also be retrogradely transported to the sensory neurons of the DRG, suggesting a role for CDF/LIF as a target-derived neurotrophic molecule for sensory neurons (25). Further support for this notion is derived from the observations that CDF/LIF rescues motoneurons in culture and in vivo (26, 27) and is expressed in embryonic muscle (19). On the other hand, we find that during development, expression of CDF/LIF mRNA and its receptor increases from P1 to adulthood in many tissues, indicating a role for the cytokine in normal homeostasis or response to injury in the adult (15). The fact that CDF/LIF mRNA is up-regulated in adult peripheral ganglia and sciatic nerve suggests that CDF/LIF induction is a general phenomenon in response to injury of the peripheral nervous system. Moreover, CDF/LIF mRNA is expressed in the adult brain at higher levels than in the neonate (19), suggesting that it might be involved in maintenance of the response to injury in the central nervous system as well. In response to intraperitoneal injection of kainic acid, the CDF/LIF mRNA increases in several brain regions (28). In addition, after cortical brain lesions, CDF/LIF mRNA reaches a peak of expression at 48 hr and returns to baseline values by 7 days after the lesion (N. Moayeri, L.R.B. and P.H.P., unpublished data).

The increase of CDF/LIF observed in vitro with sciatic nerve explants was verified with in vivo nerve transections. An increase of CDF/LIF mRNA occurs in the regions immediately adjacent to the cut site, both proximal and distal. This increase is rapid, occurring within 24 hr after injury, and is maintained for at least 2 weeks. CDF/LIF mRNA levels in the proximal-2 region do not change relative to control values at any of the times examined, demonstrating that this aspect of the injury response is highly localized. The initial rapid increase in CDF/LIF mRNA expression is somewhat similar to the induction of NGF after sciatic nerve transection. NGF mRNA levels increase dramatically within 6 hr in the regions immediately proximal and distal to the lesion site. This initial increase is followed, however, by a transient decrease between 12 and 24 hr and then by another increase in expression that peaks at 3 days (29). Although we did not examine the response of CDF/LIF 6 hr after transection, we observe a dramatic increase in mRNA expression within 24 hr. The fact that both CDF/LIF and NGF increase rapidly could be related to the observation that both cytokines are inducible by IL-1. The increase in NGF mRNA after sciatic nerve transection is due to the expression of IL-1 by macrophages that invade the lesion site (30). IL-1 also induces the expression of CDF/LIF in cultured ganglia (12). Although the low-affinity (p75) NGF receptor is up-regulated in Schwann cells in the region of degeneration (31), we find that the ligand binding subunit of the CDF/LIF receptor complex decreases somewhat after nerve injury.

The pattern of CDF/LIF induction is distinctly different from the pattern of another member of the neuropoietic cytokine family, CNTF, that has also been suggested to be involved in the response to injury. Like CDF/LIF, CNTF is normally present in Schwann cells of the sciatic nerve (32), but the lack of a signal sequence appears to prevent CNTF from being secreted. After nerve transection, CNTF mRNA levels decrease slightly at 1 day and drastically by 4 days in the regions immediately surrounding the lesion site (33, 34). CNTF protein levels only decrease slightly, however, and the protein is thought to be present in significant quantities in the extracellular space (34). The strong early induction in CDF/LIF mRNA suggests that this factor may be available very soon after damage, and this cytokine is known to be secreted (11, 12). CDF/LIF could act on macrophages that express the receptor for CDF/LIF (35) and invade degenerating nerve. CDF/LIF presumably also acts on the axons, inducing events that lead to changes in neuropeptide expression in the neuronal cell bodies (13).

The decrease in expression of the ligand binding subunit of the receptor complex, LIFR, in response to in vitro and in vivo injury is puzzling. If the decrease in LIFR mRNA expression in ganglia results in lower protein levels, then there would be potentially fewer binding sites for CDF/LIF. We (15) and others (36) have shown that both LIFR and gp130 are expressed in the neurons of sympathetic and sensory ganglia. This would indicate that the increased CDF/LIF produced in response to injury binds to receptors on the neurons to induce neuropeptide expression. If there are fewer ligand binding sites, then this increased CDF/LIF may not be as effective. In the transected sciatic nerve, the decrease in LIFR mRNA in the region surrounding the transection site continues for several days. The LIFR mRNA we are measuring in the nerve is expressed in the nonneuronal cells and whether a similar decrease is also occurring in the glia of the ganglia is not known.

Although CDF/LIF alters neuropeptide synthesis in SCG cultures, the cellular localization of the cytokine in ganglia has not been illustrated. The enormous induction of CDF/
LIF mRNA in cultured ganglia and nerve gave us the opportunity to define the cell type that expresses the cytokine. As was suggested (11), expression is prominent in nonneuronal cells. The morphology of the digoxigenin-labeled cells in the nerve resemble that of Schwann cells. Although we cannot rule out the possibility that another cell type, such as fibroblasts or macrophages, accounts for some of the staining, two lines of evidence suggest that the CDF/LIF-positive cells are glia. (i) There is a large postnatal increase in the glial cell population in sympathetic ganglia, and we have shown here that there is an enormous difference in the induction of CDF/LIF mRNA in adult vs. neonatal SCGs when the ganglia are placed in organ culture. (ii) When injured nerve and ganglia are stained with antibodies to the macrophage-specific marker MUC-102 in conjunction with in situ hybridization to CDF/LIF, the macrophage-positive cells do not colocalize with the CDF/LIF-positive cells. It is interesting to note that the CDF/LIF-positive cells in the ganglia are not the same size as those seen in the nerve.

CDF/LIF has been shown to be involved in the response to trauma and infection. This cytokine causes the induction of acute-phase proteins in liver cells (37). It is rapidly induced in bronchoalveolar fluid in response to lipopolysaccharide injection (T. R. Ulich, M.-J. Fann, P.H.P., J. Williams, B. Samal, J. delCastillo, S. Yin, K. Guo, and D. G. Remick, unpublished data), and it is present at elevated levels in synovial fluids in patients with arthritis (38). The demonstration of the dramatic induction of CDF/LIF in response to nervous system trauma provides further evidence for the role of this protein in the response to tissue damage.

We thank D. McDowell for help with tissue culture materials, K. Hatch for cloning of the GAPDH fragment, Drs. G. Krzutzberg and J. Gehrmann for the use of their MUC 102 antibody, and M.-J. Fann and M. Rao for constructive comments on the manuscript. This project was supported by grants from the Muscular Dystrophy Association (L.R.B.) and the National Institute of Neurological Disorders and Stroke (J.B.B.).